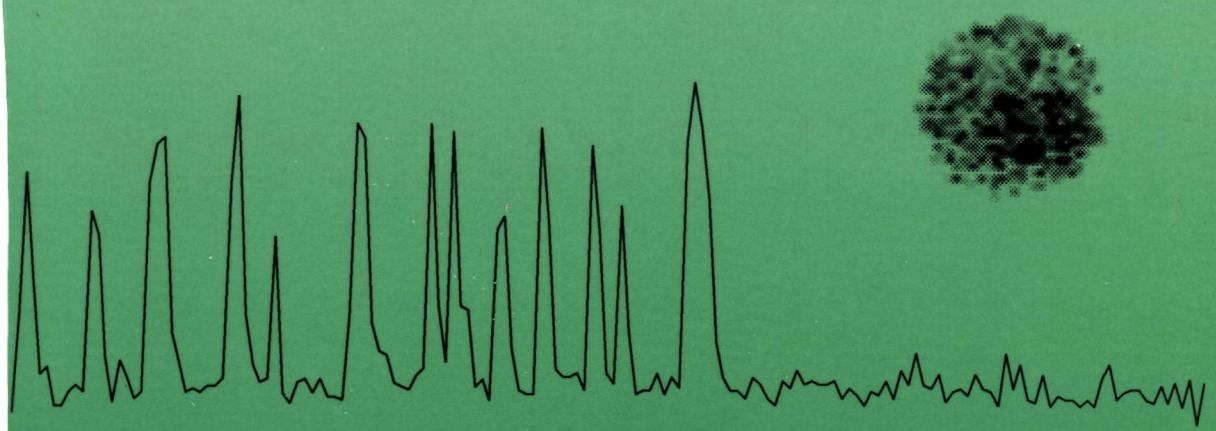
CALCIUM DYNAMICS IN MELANOTROPE CELLS OF *XENOPUS LAEVIS*



Wim Scheenen

CALCIUM DYNAMICS IN MELANOTROPE CELLS OF *XENOPUS LAEVIS*

een wetenschappelijke proeve
op het gebied van de Natuurwetenschappen

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Wilhelmus Johanna Josephina Margaretha Scheenen

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Promotor: Prof. Dr. E.W. Roubos

Co-promotores: Dr. B.G. Jenks
Dr. P.H.G.M. Willems

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voor mijn ouders

Illustratie omslag Gedigitaliseerde opname van een melanotrope cel, met een bijbehorende trace van een tijdgerelateerde Ca^{2+} dynamiek

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

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CONTENTS

GENERAL INTRODUCTION

Intracellular signal transduction

For a multicellular organism it is important that cells communicate with each other in order to guarantee proper functioning of the cells. Communication between cells takes place via secretion of signaling molecules, the first messengers, and the subsequent binding of these molecules to receptors on the target cell. Once binding has taken place, the signal will be transduced into a message for the cell to control one or more cellular processes. In the case of cell surface receptors, this signal transduction event can involve a direct control of transmembrane ion channels or membrane-bound enzymes, or activation of a G-protein, that subsequently activates or inactivates either ion channels or enzymes which produce intracellular messenger molecules, referred to as second messengers. In a wide variety of cell types emphasis has been placed on the importance of the Ca^{2+} ion [1] as an intermediate second messenger molecule that can regulate several cellular processes. Already in 1964 the importance of Ca^{2+} for the secretory process of a cell was described [2] and to date it is known that elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) may induce gene expression [3]. To gain further insight into the role of Ca^{2+} in the secretory process, melanotrope cells of the South African clawed toad *Xenopus laevis* were used as the experimental object.

Melanotrope cells of Xenopus laevis

The amphibian *Xenopus laevis* is capable of adapting its skin color to the light intensity of its environmental background. In this process of background adaptation melanotrope cells of the pituitary pars intermedia play a pivotal role [4]. These cells produce α-melanophore stimulating hormone (α-MSH) which is derived from the precursor protein proopiomelanocortin. This hormone causes dispersion of the pigment melanin in skin melanophores, which makes the skin appear dark. In order to regulate its skin color, the animal needs a rigorous control of α-MSH secretion. The regulation of the melanotrope cells of *Xenopus* has been studied in detail [4]. At least three neural inhibitors of secretion (secreto-inhibitors) have been described, namely dopamine, neuropeptide Y (NPY) and γ-aminobutyric acid (GABA). For GABA two receptor subtypes have been pharmacologically characterized, a $GABA_A$ receptor, which is a chloride channel, and a $GABA_B$ receptor, which, like the D_2 receptor, is a G-protein-coupled receptor [5]. All three secreto-inhibitors coexist in nerve terminals, that make synaptic contacts with the melanotrope cells [6]. These terminals are from cell bodies located in the hypothalamic suprachiasmatic nucleus [7,8]. Apart from the secreto-inhibitors, spontaneous *in vitro* secretion of

α-MSH from *Xenopus* melanotropes can be further stimulated by the secreto-stimulators corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) [4]. These peptides originate from neurons in the hypothalamic magnocellular nucleus [7,8] that have nerve endings in the pituitary pars nervosa. It has been hypothesized that these factors diffuse to the pars intermedia in order to stimulate α-MSH secretion [4].

Signal transduction pathways in Xenopus melanotropes

Apart from studies on the hypothalamic regulation of α-MSH release, a great deal of attention has been paid to second messenger pathways operating in melanotropes. Since melanotropes are excitable cells, intracellular processes after receptor activation are not restricted to second messenger molecules like c-AMP but also involve membrane events. A good example ofsuch an involvement is the action of the inhibitory $GABA_A$ receptor [9]. Activation of this receptor leads to opening of a membrane chloride channel. In melanotrope cells of the frog *Rana ridibunda* it has been found that chloride channel opening leads to chloride influx and thus to hyperpolarization of the cell membrane [10]. This in turn causes inhibition of α-MSH secretion. Regarding second messengers that can be activated in melanotropes most attention has been paid to c-AMP. The inhibitory dopamine and $GABA_B$ receptors induce a decrease in c-AMP levels [5] whereas activation of a CRH receptor increases the c-AMP level [5]. Until now little is known about the action of TRH. In melanotropes of *Rana ridibunda* TRH stimulates the production of inositol-(1,4,5)-trisphosphate (IP_3) [11]. In *Xenopus* melanotropes IP_3 might also play a role in regulating cellular activity, since activation of melanotropes during adaptation to a black background leads to an increase in the level of inositol phosphates [12].

Although much research has been carried out on the external regulation of melanotrope cells and the subsequent second messenger pathways, little information is available on the role of the Ca^{2+} ion, except that it has been found that α-MSH secretion depends on the presence of Ca^{2+} [13]. Research on the Ca^{2+} dynamics in *Xenopus* melanotrope cells forms the subject of this thesis.

As described in the next sections there are two sources of Ca^{2+} for intracellular signaling, one involving Ca^{2+} entry from the extracellular environment and the other involving Ca^{2+} release from intracellular stores like the endoplasmic or sarcoplasmic reticulum [1,14].

Ca^{2+} entry from the extracellular environment

Extracellular Ca^{2+} can enter cells through voltage-operated Ca^{2+}-channels (VOCC) or receptor-operated Ca^{2+} channels (ROCC). All excitable cells possess voltage-operated Ca^{2+} channels and intensive research has made clear that several types of these channels can occur in one and the same cell. Based on the voltage characteristics, low-voltage activated (LVA) and high-voltage activated (HVA) Ca^{2+} channels can be distinguished [15]. The LVA channels rapidly inactivate, leading to a transient Ca^{2+} current and are therefore referred to as T-type channels [15]. Pharmacology of the HVA channels has shown several types of HVA channels. Some are sensitive to derivatives of dihydropyridines, like nifedipine. These channels have slow inactivating kinetics, leading to long-lasting Ca^{2+} currents and are, therefore, called L-type Ca^{2+} channels [15,16]. Channels which exhibit neither of both inactivating kinetics are predominantly found in neurons and have been called N-type Ca^{2+} channels [15,17]. N-type channels are sensitive to a toxin of the snail *Conus geographicus*, ω-conotoxin GVIA [18].

The second Ca^{2+} entry pathway involves ROCC, that are not sensitive to membrane depolarizations but require direct activation by a receptor or intracellular ligand. In contrast to VOCC, little is known about ROCC. A receptor may bind directly to a Ca^{2+}-channel or via an intermediate G-protein. Also $[Ca^{2+}]$ in the endoplasmic reticulum can determine Ca^{2+} entry, the so-called 'capacitative Ca^{2+} entry' [19]. For most VOCC it is known that they can be activated by second messenger-induced phosphorylation [20] and then they can act as ROCC.

Ca^{2+} release from intracellular stores

Cleavage of the membrane phospholipid phosphatidyl 1,4-bisphosphate by phospholipase C (PLC) leads to the formation of the second messengers IP_3 and 1,2-diacylglycerol (DAG) [21]. Activation of PLCβ occurs through the G-protein G_q; no conclusive evidence has been found for an action of an inhibitory G-protein on this enzyme [22]. The IP_3-receptor is located on the endoplasmic reticulum and forms a tetrameric Ca^{2+} channel [23]. Binding of IP_3 initiates channel opening which will lead to an increase in $[Ca^{2+}]_i$ by release of Ca^{2+} stored in the endoplasmic reticulum. IP_3 can only bind to its receptor in the presence of low $[Ca^{2+}]_i$, whereas high $[Ca^{2+}]_i$ inhibits the binding of IP_3 [24]. The process whereby Ca^{2+} coagonizes with IP_3 in inducing Ca^{2+} release is called IP_3-sensitive calcium-induced calcium-release (IP_3R-CICR).

Apart from the IP_3-induced Ca^{2+} release, Ca^{2+} itself can also induce release from IP_3-insensitive intracellular Ca^{2+} stores. This process is well described for the sarcoplasmic reticulum of muscle cells, but it is also found in other cell types [25-27]. Since the Ca^{2+}-sensitive Ca^{2+} release channel is also sensitive to ryanodine, this type of Ca^{2+} release has been termed ryanodine-sensitive calcium-induced calcium-release (RYR-CICR).

Intracellular Ca^{2+} oscillations

With the development of sensitive, dynamic techniques for imaging Ca^{2+} changes in single cells it became clear that in many cell types the $[Ca^{2+}]_i$ transiently rises above a baseline, the so called Ca^{2+} oscillations. The increase in $[Ca^{2+}]_i$ during Ca^{2+} oscillations can be seen as Ca^{2+} waves going through the cytoplasm. One possible mechanism for initiation of Ca^{2+} oscillations is through a spatiotemporal relationship of Ca^{2+} release from intracellular stores. Several mechanisms have been proposed by which an intracellular oscillator can lead to Ca^{2+}-oscillations and can include a RYR-CICR or a IP_3R-CICR [for reviews see 1,28]. For both the RYR-CICR and the IP_3R-CICR a complex feedback system exists, in which opening and closing of the Ca^{2+} release channel is sensitive to both the $[Ca^{2+}]$ in the cytoplasm and in the intracellular stores. As a result, $[Ca^{2+}]$ will oscillate in both the cytoplasm and the intracellular stores. Another mechanism by which oscillations can be generated is through periodic opening of VOCC [29]. This type of oscillations, initiated by a membrane oscillator, is found in several cell types including pituitary pars distalis cells [29].

Ca^{2+} oscillations can be either spontaneous or induced by agonists [1,29]. In smooth muscle cells and several muscle cell lines the existence of spontaneous Ca^{2+}-oscillations have been described. These oscillations occur through RYR-CICR [30,31]. In rat and bovine chromaffin cells, also displaying spontaneous Ca^{2+} oscillations, extracellular Ca^{2+} is not directly involved in oscillation induction and in these cells both a RYR-CICR and IP_3R-CICR seem to be present [32,33]. Agonist-induced oscillations have been found in a variety of cell types, including several pars distalis cell types [29], pancreatic acinar cells [1], avian exocrine gland cells [34] and parotid acinar cells [35]. For pancreatic acinar cells two types of Ca^{2+} oscillations have been described, depending on the agonist applied. One type is located in the apical region of the cell, and is primarily dependent on extracellular Ca^{2+}. The other type occurs through the entire cell (global) and depends on an interaction between RYR-CICR and IP_3R-CICR [1,36].

Although a great deal of research has been conducted on the mechanisms by which $[Ca^{2+}]_i$ can increase as a result of cell stimulation, for secretory cells no general consensus exists on the physiological role of Ca^{2+} oscillations *versus* sustained elevations in baseline $[Ca^{2+}]_i$. While many studies suggest that Ca^{2+} oscillations are associated with the secretory pathway [28,37] others suggest that Ca^{2+} oscillations are important for gene regulation and that sustained elevations in basal $[Ca^{2+}]_i$ are important in setting the level of secretion [38]. The aim of the studies described in this thesis was to characterize Ca^{2+} dynamics in melanotrope cells of *Xenopus laevis*. An understanding of how this cell type transduces its complex multiple neural input into an output, and of the role Ca^{2+} plays in this process, will improve our knowledge of the functioning of these cells and of Ca^{2+} signaling in general.

To obtain a better view of the relationship between Ca^{2+} entry mechanisms and secretion, electrophysiological properties of *Xenopus* melanotropes have been investigated in correlation with hormone secretion. Based on the experiments described in Chapter 1 it is concluded that *Xenopus* melanotropes possess spontaneous electrical activity and two types of HVA Ca^{2+}-channels. Of these channels, only the ω-conotoxin GVIA-sensitive Ca^{2+}-channel seems to be involved in Ca^{2+} entry associated with secretion. Studies on individual melanotropes, using Ca^{2+} imaging methods, were performed to obtain insight into the nature of Ca^{2+} dynamics (Chapter 2). It was found that a vast majority of the cells displayed spontaneous Ca^{2+} oscillations initiated by influx of extracellular Ca^{2+} through ω-conotoxin GVIA-sensitive Ca^{2+} channels. Subsequently, experiments were performed to investigate a possible role of spontaneous Ca^{2+} oscillations in hormone secretion. In Chapter 3 an overview is given of the effects of secreto-inhibitors and secreto-stimulators on spontaneous Ca^{2+} oscillations. Chapter 4 presents a more detailed study on the mechanism by which NPY inhibits oscillatory and secretory activity. From the results described in Chapters 3 and 4 it is hypothesized that Ca^{2+} oscillations are an important driving force for secretion. The mode of action of the secreto-inhibitors was further investigated in Chapter 5, where the effects of membrane depolarizations on Ca^{2+} dynamics were examined in inhibited cells. The secreto-inhibitors apparently inhibit through different mechanisms, the two extremes being baclofen (causing a reversible inhibition) and dopamine (causing an irreversible inhibition). In Chapter 6 spatiotemporal aspects of the oscillations were investigated, using confocal laserscanning microscopy. Evidence is provided that spontaneous Ca^{2+}oscillations occur in the cytoplasm as well as in the nucleus. These two cellular compartments are coupled to each other with respect to the oscillations. After an oscillation is initiated by a membrane oscillator, its propagation through

the cell most likely takes place through an IP_3-sensitive CICR; the amplitude of the intranuclear Ca^{2+} rise can be modulated by intracellular ATPase activity. In the General Discussion of this thesis a model for control and significance of Ca^{2+} dynamics in the melanotrope cell of *Xenopus laevis* is presented.

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SECRETION OF α-MSH FROM *XENOPUS LAEVIS* MELANOTROPES DEPENDS ON CALCIUM INFLUX THROUGH ω-CONOTOXIN-SENSITIVE VOLTAGE-OPERATED CALCIUM CHANNELS

With Bruce G Jenks, Harry P. de Koning, Hubert Vaudry and Eric W. Roubos
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The secretory activity of endocrine cells largely depends on the concentration of free cytosolic calcium. We have studied the mechanisms that are involved in supplying the calcium necessary for the secretion of α-melanophore-stimulating hormone (α-MSH) from melanotrope cells in the pituitary intermediate lobe of the amphibian *Xenopus laevis*. Using whole-cell voltage clamp, high-voltage activated calcium currents were observed, with a peak current between 0 and +20 mV. Two types of Ca^{2+}-currents appeared, depending on the experimental setup. An inactivating current, which was observed after a 10 msec depolarizing prepulse, resembled currents through N-type channels as it was clearly inhibited by 1 μM ω-conotoxin. The second type was a non-inactivating current, which was blocked up to 50 % by 1 μM nifedipine, indicating its L-type nature. Only a small component of this inactivating current could be blocked by ω-conotoxin. No evidence was found for the presence of transient, low-voltage activated currents.

The spontaneous secretion of α-MSH from superfused neurointermediate lobes was dependent on extracellular calcium, as low calcium conditions (10^{-4} - 10^{-8} M) rapidly inhibited this process. Under these conditions, secretion was not affected by depolarizing concentrations of potassium chloride. The calcium ionophore A23187 increased secretion under low calcium conditions, but had no effect on spontaneous α-MSH release. Treatment with $CoCl_2$, a blocker of calcium channels, strongly inhibited the secretory process. These results suggest that spontaneous α-MSH release depends on influx of calcium through voltage-operated calcium channels. Nifedipine did not affect spontaneous secretion from lobes, nor did it affect potassium-induced α-MSH secretion from dispersed melanotropes. Also BAY-K8644, a specific agonist of L-type channels, did not influence α-MSH release, neither under normal nor under low calcium conditions. On the other hand, ω-conotoxin dose dependently inhibited α-MSH release, to a maximum of 65 % at a concentration of 5μM, and inhibited potassium-induced secretion by 40 %. Thapsigargin, an agent that mobilises calcium ions from intracellular stores, had no effect on spontaneous α-MSH release under normal or low calcium conditions. From these results it is concluded that the spontaneous release of α-MSH by melanotropes of *X. laevis* is effectuated by calcium influx through ω-conotoxin-sensitive, voltage-operated N-type calcium channels and that mobilization of calcium from intracellular stores does not play a major role in the regulation of this release.

The amphibian melanotrope cell is involved in the physiological process of background adaptation. It functions as a neuroendocrine transducer cell integrating multiple neural inputs into an endocrine output, namely the secretion of α-melanophore-stimulating hormone (α-MSH) [1]. For the melanotrope cell of the toad *Xenopus laevis*, the neural inputs include dopamine, γ-aminobutyric acid and neuropeptide Y, which inhibit α-MSH secretion, and corticotropin-releasing hormone and thyrotropin-releasing hormone, which stimulate secretion [2,3]. The mechanisms by which these regulators cooperate to control secretion are largely

unknown. It is likely that the factors act through transmembrane signaling mechanisms that, directly or indirectly, influence the intracellular concentration of free calcium ($[Ca^{2+}]_i$) thus regulating secretion [4]. Thomas et al. [5] have correlated increased $[Ca^{2+}]_i$ with increased membrane capacitance reflecting stimulated release by exocytosis. An understanding of the mechanisms controlling changes in $[Ca^{2+}]_i$ will enhance our insight in the process of integration of multiple inputs by (neuroendocrine) secretory cells. A rise in $[Ca^{2+}]_i$ can occur through mobilization of calcium from intracellular stores, a process regulated by the second messenger inositol 1,4,5-triphosphate [6,7]. Another potential source of Ca^{2+} for supporting the secretory process is the entry of extracellular calcium (Ca^{2+}_e) through voltage-operated calcium channels (VOCC) [8,9,10,11]. Free calcium levels may be determined by a combination of both mechanisms, the contribution of each depending on the nature of neural inputs to the neuroendocrine cell.

The presence of VOCC in melanotrope cells has been studied in several amphibian and mammalian species [12,13,14,15,16,17]. It appears that melanotropes possess high-voltage operated Ca^{2+} channels. In addition to these types, mammals show low-voltage activated calcium channels [15,16,18]. Relatively little attention has been paid to the possible involvement of calcium channels in the secretory process. A minor role for L-type channels in supporting the secretory process has been reported for mouse and frog melanotrope cells [19,20]. The present paper concerns a multidisciplinary study, involving electrophysiological, pharmacological and superfusion techniques, on the characteristics of VOCC in melanotrope cells of the amphibian *X. laevis* and, in particular, on the possible role of these channels in supporting the secretion of α-MSH. *Xenopus* melanotropes have been chosen for this study because of their well known secretory characteristics [2]. These cells are primarily under inhibitory regulation and display high rates of spontaneous secretion when placed *in vitro*. The results show that while the melanotropes have both ω-conotoxin-sensitive N-type channels and nifedipine-sensitive L-type channels, only N-type channels are associated with the secretory process.

MATERIALS AND METHODS

Animals

Young-adult *Xenopus laevis* were taken from laboratory stock and adapted to a black background for three weeks under continuous illumination, at 22 °C The animals were fed weekly with beef heart

Preparation of isolated cells

Isolation of melanotrope cells was performed as described previously [37] In short, after perfusing the animal with *Xenopus* Ringer's solution, containing 112 mM NaCl, 15 mM

Ultral-HEPES (Calbiochem, La Jolla CA, USA, pH 7 4), 2 mM KCl and 2 mM $CaCl_2$, to remove blood cells, neurointermediate lobes were dissected and incubated for 45 min in Ringer's solution without $CaCl_2$, to which 0 25 % (w/v) trypsin (Gibco, Renfrewshore, UK) had been added Cells were subsequently dispersed in Leibovitz's L15 medium which contained 10 % fetal calf serum (Gibco) by gentle trituration of the lobes with a siliconized Pasteur's pipette The medium was adjusted to *Xenopus* osmolality (L15 Milli Q = 2 1) After washing, the cells were plated on cover slips coated with poly-L-lysine (Sigma, St Louis, MO, USA, Mw > 300 kD) at a density of about 10,000/slide, and cultured for 3 days, at 22 °C For superfusion experiments with isolated melanotropes, cell suspensions were mixed with Cytodex-3 beads (Pharmacia, Uppsala, Sweden) to prevent reaggregation [45], and cultured for 3 days, at 22 °C

Electrophysiological studies

Barium currents through calcium channels were recorded by using the whole-cell patch clamp configuration, at 22 °C, according to Hamill *et al* [46] Half an hour before electrophysiological recording, the culture medium was replaced with a bathing solution containing 112 mM NaCl, 20 mM tetraethylammonium (TEA)-Cl, 10 mM $BaCl_2$, 15 mM HEPES and 1 µM tetrodotoxin, pH was adjusted to 7 4 using TEA-OH Soft glass patch electrodes of 2-4 MW resistance were made with a vertical pipette puller (David Kopf Instruments, USA) and heat-polished on a microforge Electrodes were filled with a solution containing 100 mM CsCl, 20 mM TEA-Cl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM ethylenoglycoltetraacetic acid (EGTA) and 10 mM HEPES, pH was adjusted to 7 4 using TEA-OH Current signals were recorded with an EPC-7 patch-clamp amplifier (List Electronics, FRG) and digitally stored on audiotape at a sampling rate of 44 kHz using a pulse code modulator (Sony, Japan) Depolarizing test pulses were applied with a programmable stimulator (Biologic, France) The holding potential was -80 mV Capacitive currents were corrected using the analog circuitry on the amplifier Off-line data were digitized at sampling rates of 100 µs per point using a Labmaster TL-1 DMA motherboard interfaced with an IBM-compatible 80386 microprocessor-based computer The data were analyzed using pClamp 5 5 1 software (Axon Instruments, Burlingame, CA, USA) Leakage was determined from currents evoked by hyperpolarizing pulses to -160 mV Nifedipine (Sigma, St Louis, MO, USA) and ω-conotoxin GVIA (Peninsula Laboratories, Belmont CA, USA) were added to the bathing solution from a large tipped pipette, yielding a final concentration of 1 µM When action potentials were recorded in the whole cell configuration, non-selective pipette and bath solutions were used Medium in the pipette contained 100 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM EGTA and

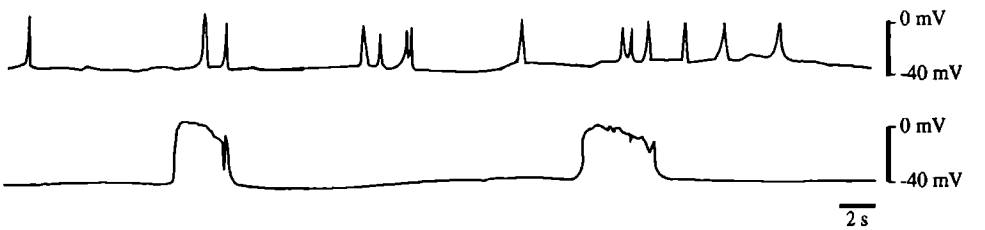

Figure 1: Whole-cell recording of spontaneous electrical activity in a melanotrope cell of *Xenopus laevis* Membrane potential was -38 mV (N=11) **A**, Spikes of short duration (N=4) **B**, Plateau potentials lasting for 1 to 15 seconds (N=9)

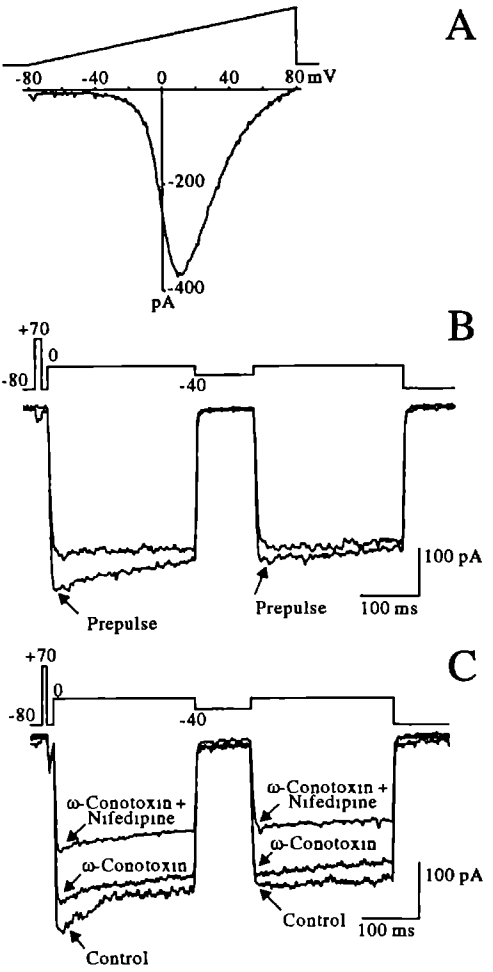

Figure 2: Whole-cell voltage clamp recording of barium current through calcium channels **A**, Current-voltage (I/V) relation of ramp potential, ranging from -80 mV to +80 mV. Peak currents occur between 0 and +20 mV; in this example +10 mV. **B**, Effect of giving a prepulse on barium currents. There is no inactivation of the calcium currents, since peak current and the current at the end of the test pulse hardly differ. When a strong depolarizing prepulse to +70 mV is given at the beginning of the pulse protocol a clear inactivating component of the barium current appeared (lower trace, indicated by prepulse) **C**, Effects of ω-conotoxin and nifedipine on barium currents The pulse protocol is the same as in Fig. 3. ω-Conotoxin strongly inhibited the inactivating current component and part of the non-inactivating component. Subsequent adding nifedipine reduced the non-inactivating component to 50 % of the initial value.

10 mM HEPES (pH 7.4). Non-selective extracellular medium (IM) contained 112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$ and 15 mM HEPES (pH 7.4).

In vitro superfusion

Neurointermediate pituitary lobes were dissected out and placed into 10 µl superfusion chambers. Four such chambers were superfused simultaneously with incubation medium (IM), consisting of 112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 15 mM HEPES (pH 7.4), 2 mg/ml glucose, 0.3 mg/ml bovine serum albumin and 1 mg/ml ascorbic acid, at a rate of 1.5 ml/h, at 22 °C In order to establish a stable release, lobes were superfused for at least 75 min (10 fractions of 7.5 min) with IM before test substances (or different calcium conditions) were introduced Basal (spontaneous) α-MSH release was defined as the average secretion in the three fractions preceding the first application Drugs tested were thapsigargin (Calbiochem, La Jolla, CA), A23187 (free acid, Sigma), BAY K8644 (Miles Pharmaceuticals, West Haven, USA), nifedipine, and ω-conotoxin GVIA. For administration protocols of drugs used, see Results. BAY K 8644 and nifedipine were dissolved in ethanol (maximum concentration 0.1%,

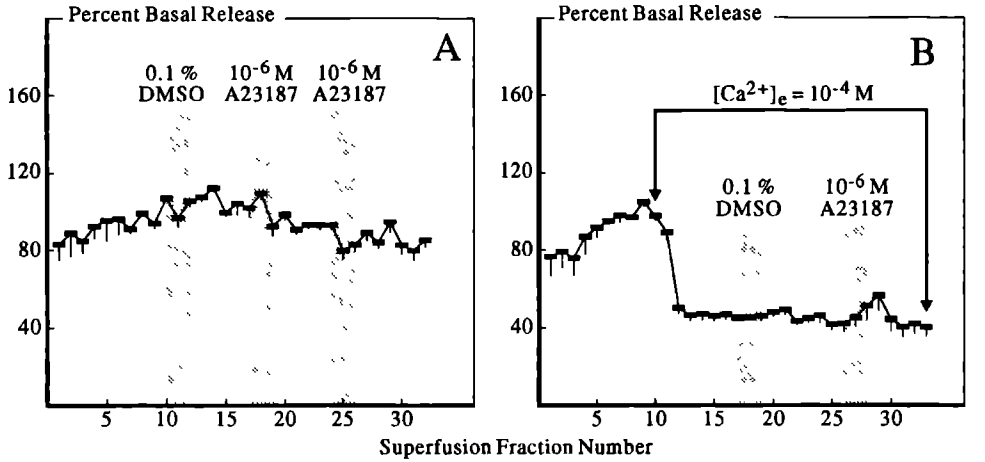

Figure 3: Effect of A23187 on α-MSH release. **A**, adding A23187 during basal conditions (10^{-6} M) had no significant effect on basal release (9.6 ± 4.4 and 2.5 ± 6.5% inhibition, respectively). Data are the average of eight experiments. **B**, adding A23187 during low Ca^{2+} conditions (10^{-4} M) stimulated α-MSH release by 19.1 ± 7.7% ($p<0.05$). The solvent of A23187, 0.1% DMSO, had no effect on secretion.

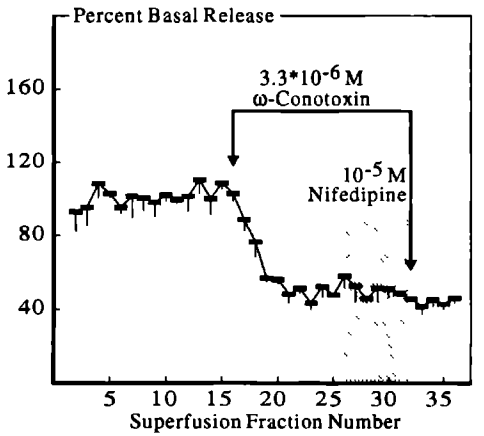

Figure 4: Effect of ω-conotoxin (3.3×10^{-6} M) and subsequent addition of nifedipine (10^{-5} M) on spontaneous secretion from neuro-intermediate lobes.

v/v). A23187 (10^{-6} M) was dissolved in IM containing 0.1% dimethyl sulfoxide. In experiments where various concentrations of KCl and/or $CaCl_2$ were applied, osmolality of the medium was kept constant by adjusting the NaCl concentration. Low levels of $CaCl_2$ were buffered with EGTA.

Radioimmunoassay

Radioimmunoassay for α-MSH was performed as described previously, using an antiserum raised in our laboratory [47], which has equal affinities for acetylated and non-acetylated α-MSH. Cross-reactivities with ACTH (1-24) and ACTH (1-39) were lower than

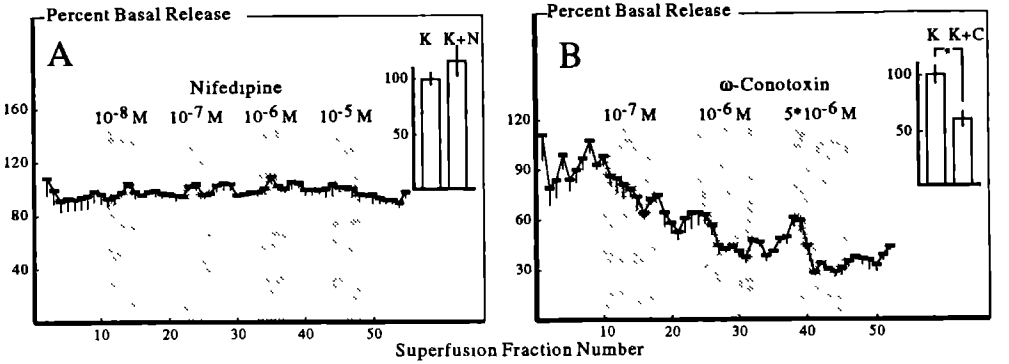

Figure 5: Dose-response of specific blockers of L- and N-type calcium channels. **A**, None of the nifedipine concentrations tested significantly affected secretion. Insert shows the absence of effect of nifedipine on K^+-induced secretion from dispersed melanotropes (N=4).
B, ω-Conotoxin progressively reduced α-MSH release to 70.8 ± 1.6% (10^{-7} M, $p<0.001$), 42.1 ± 3.2% (10^{-6} M, $p<0.01$) and 31.1 ± 2.2% (5 x 10^{-6} M, $p<0.001$) of basal level (average secretion in the last four fractions of each ω-conotoxin administration). ω-Conotoxin inhibited the K^+-induced secretion from dispersed melanotropes by 40 % ($p<0.05$, N=4, insert). The K^+-concentration used to induce secretion was 60 mM.

0.01%. Bound and free antiserum were separated by polyethylene-glycol/ovalbumin precipitation. Detection limit was 2 pg α-MSH per 50 µl sample. Superfusion fractions were assayed in duplicate.

Calculations and statistics

Results of superfusion experiments are shown as the average of four experiments ± SEM, unless stated otherwise. Percentages of stimulation and of inhibition of α-MSH secretion in response to experimental treatment were calculated on the basis of integration of the respective peak areas in the graphs, using three fractions just before and during treatment. Data were tested for significance using the paired Student's T-test. A p-value <0.05 was considered to indicate statistical significance.

RESULTS

Patch-clamp recordings

In non-selective medium, melanotropes had a membrane resting potential of 38.7 ± 4.9 mV (mean ± S.D., N=11). All cells studied under current-clamp displayed spontaneous electrical activity (Fig. 1). Two types of activity were observed. One consisted of a series of fast depolarizations resembling action

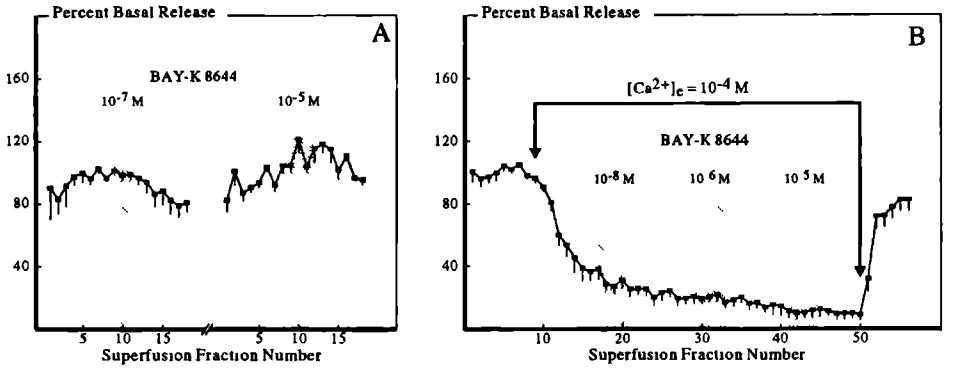

Figure 6: Absence of effect of BAY-K8644 on secretion from superfused neurointermediate lobes under normal (**A**) and low Ca^{2+}_e (**B**) conditions

potentials (Fig. 1A, N=4). Another type of electrical activity consisted of prolonged depolarizations ('plateau potentials') with a duration of 1 to 15 seconds (Fig. 1B, N=9). In 2 out of 9 cells, both types of electrical activity were observed. In an initial series of whole cell voltage clamp experiments, where barium currents were evoked by a voltage ramp ranging from -80 mV to +80 mV, evidence was found for the occurrence of high-voltage activated calcium channels in melanotropes (Fig. 2A). The barium current had a threshold of -20 mV and the peak current was observed between 0 and +20 mV (N=6). When cells were depolarized using a double depolarizing protocol, as shown in Figure 2B, no inactivating currents were observed in response to either of the two depolarizing pulses. However, when a strong 10 msec depolarizing pulse to +70 mV (prepulse) was given before starting the protocol, an inactivating barium current appeared in the first depolarizing pulse from -80 to 0 mV (Fig. 2B). To study the effect of ω-conotoxin and nifedipine on both inactivating and non-inactivating barium currents, a prepulse was always included in the rest of the experiments. Adding 1 μM ω-conotoxin, an antagonist of N-type VOCC, to the bathing solution partially blocked the inactivating component occurring during the first depolarizing pulse as well as a small component of the non-inactivating component (Fig. 2C, N=5). Subsequent addition of 1 μM nifedipine reduced the remaining non-inactivating current by about 50 % of the initial value (Fig. 2C).

Effects of low $[Ca^{2+}]_e$ on spontaneous and A23187-induced α-MSH release

Application of medium with a low calcium concentration reduced the rate of α-MSH secretion from superfused neurointermediate lobes. This effect was clearly observed within 2 superfusion fractions (15 min) and was maximal after 3-4 fractions. The calcium ionophore A23187 (10^{-6} M) had no effect on

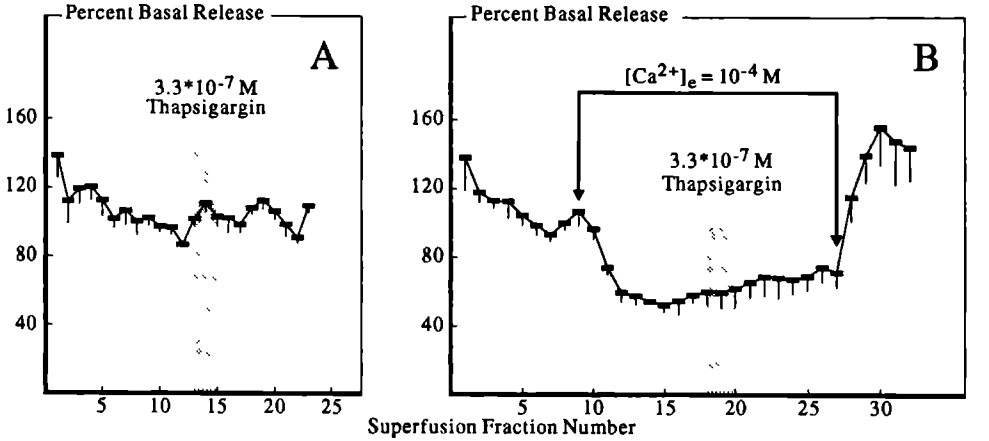

Figure 7: Absence of effect of thapsigargin on α-MSH release from superfused neuro-intermediate lobes during basal (**A**) and low calcium (**B**) conditions.

spontaneous secretion but slightly increased α-MSH release under low calcium conditions (10^{-4} M) by 19.1 ± 7.7 % ($p < 0.05$, Fig. 3A,B).

Effect on α-MSH secretion of treatments that affect voltage-operated calcium channels

Administration of 0.1 to 5 mM $CoCl_2$, a general calcium channel blocker, produced an inhibition of α-MSH release (Data not shown). ω-Conotoxin (3.3 x 10^{-6} M) caused a slow but clear decrease in α-MSH secretion and addition of nifedipine during ω-conotoxin had no effect (Fig. 4). Nifedipine alone (10^{-8} - 10^{-5} M) had no effect on α-MSH secretion (Fig. 5A) nor did it influence K^+-induced secretion from dispersed melanotropes. ω-Conotoxin induced a dose-dependent inhibition of α-MSH release (Fig. 5B). The inhibition was slow in onset and not rapidly reversible and subsequent pulses with increasing concentrations of ω-conotoxin had a cumulative effect, causing about 65 % inhibition of secretion at $5*10^{-6}$ M. Moreover, ω-conotoxin inhibited K^+-induced secretion from dispersed melanotropes. BAY K8644 also had no effect on α-MSH release under normal and low calcium conditions, at concentrations up to 10^{-5} M (Fig. 6A,B).

The effect of thapsigargin on a-MSH secretion

Thapsigargin (3.3 x 10^{-7} M), an agent known to mobilize Ca^{2+} from intracellular Ca^{2+} stores by inhibiting microsomal Ca^{2+}-ATPase activity, had no

effect on α-MSH secretion under normal or low (10^{-4} M) calcium conditions (Fig. 7A,B).

DISCUSSION

In the present study, we have investigated to what extent calcium influx through VOCC and calcium mobilization from intracellular stores are involved in spontaneous secretion of α-MSH from *Xenopus* melanotrope cells. A possible mechanism by which cytosolic calcium levels are controlled in *Xenopus* melanotropes is through modulation of VOCC in the plasma membrane. The involvement of such channels is plausible because melanotropes of various species display spontaneous action potentials causing sufficient depolarization to induce VOCC opening [12,13,14,21]. Such a phenomenon was first described in GH3 cells by Kidokora [22]. We now show that melanotrope cells of *Xenopus* also display spontaneous electrical activity. Moreover, we demonstrate the presence of high voltage-activated calcium currents in these cells. These currents might be activated during transient or longlasting depolarizations to provide the calcium necessary for inducing spontaneous α-MSH release. No evidence was found for low voltage-activated channels (T-type) that give rise to a rapidly inactivating current [10,23] and thus their presence in *Xenopus* melanotropes remains to be established. The observation that Ca^{2+} currents display two distinct types of inactivation kinetics, which can selectively be displayed by applying a strong depolarizing pulse of short duration as described for chromaffin cells [24,25], suggests that *Xenopus* melanotrope cells, like those of rat [16,17,18], pig [15] and frog [14], possess a mixed population of VOCC. In view of their voltage-dependence and kinetic characteristics, the occurrence of both inactivating and non-inactivating currents suggests the presence of N-type and L-type currents, respectively. Our pharmacological characterization of these two voltage-dependent kinetics, showing that the inactivating current, representing N-type VOCC, can be blocked by ω-conotoxin whereas part of the non-inactivating current, representing L-type VOCC, is particularly sensitive to nifedipine, further establishes the presence of at least two high voltage-activated Ca^{2+} channel subtypes in *Xenopus* melanotropes. It is interesting that not all Ca^{2+} currents were blocked with the combined ω-conotoxin-nifedipine treatment. This raises the possibility for the existence of other high-voltage activated Ca^{2+} channels on *Xenopus* melanotropes, similar to the situation reported for rat melanotropes, where recently the existence of a P-type channel has been suggested [26].

The finding of at least two types of Ca^{2+} channels on *Xenopus* melanotropes was the motivation to examine the potential role of extracellular Ca^{2+} and

VOCC on the secretory process. The role of calcium in spontaneous α-MSH secretion from *Xenopus* melanotropes was the subject of an earlier study where it was shown that secretion from dispersed cells can be stimulated by a depolarizing pulse of K^+, an action that was blocked in Ca^{2+} free medium [27]. The secretory process of melanotrope cells appears to depend on extracellular Ca^{2+} (Ca^{2+}_e), as reported for mammalian melanotropes [28,29,30,31] and for those of lower vertebrates [20,32,33]. In the present study, the relatively rapid inhibition of secretion as a result of lowering the external calcium concentration indicates that spontaneous α-MSH secretion is stimulated by influx of Ca^{2+}_e. The high spontaneous (*in vitro*) release rate of α-MSH found for melanotrope cells of many species including *Xenopus*, most likely is the result of spontaneous membrane depolarizations mediated by inward currents of Na^+ and Ca^{2+} [12, 13,14,15,21]. The observation that $CoCl_2$ treatment causes a rapid inhibition of α-MSH secretion indicates that the influx of Ca^{2+}_e required for strong α-MSH secretion is mediated by VOCC. While $CoCl_2$ acts primarily on calcium channels [34] it may also have less specific effects on intracellular calcium-binding proteins [35] and it is not selective for a specific type of VOCC. Therefore, the effects of specific agonists and antagonists of VOCC were examined. The involvement of N-type channels in melanotrope secretory activity clearly appears from the fact that ω-conotoxin dose-dependently inhibited α-MSH release. There are some reports that ω-conotoxin also acts on L-type VOCC [for reviews see refs 10 and 11]. Since the blocker of the L-type VOCC, nifedipine, had no effect on α-MSH secretion, calcium influx through L-type channels does not seem to be essential for spontaneous release. We therefore conclude that the ω-conotoxin-sensitive channel examined in the present study is of the N-type. Since nifedipine only binds to L-type VOCC when the channel is in the open state, a strong K^+-induced depolarization was provided during nifedipine treatment to cells in suspension, and, here too, nifedipine had no effect on the secretory response. In contrast, when a K^+-depolarization was performed during ω-conotoxin treatment, the secretory response to potassium was blocked. These observations again indicate that N-type VOCC and not L-type VOCC are involved in α-MSH secretion from *Xenopus* melanotropes. Also the failure of BAY-K 8644 (a specific agonist of L-type VOCC) to affect α-MSH release under normal and low calcium conditions argues against a role of L-type channels in inducing spontaneous release. Even a minor role for this class of calcium channels could not be demonstrated because nifedipine also had no effect during ω-conotoxin treatment. Such an effect would have been expected if L-type channels, not blocked by ω-conotoxin, would provide the Ca^{2+} necessary for the residual secretion observed during ω-conotoxin treatment. The absence of a role for L-type VOCC in supporting α-MSH secretion from *Xenopus* melanotropes

contrasts to the situation in melanotrope cells of the frog *Rana ridibunda* [20], the rat [36] and the mouse [12,19], where dihydropyridine-sensitive L-type channels do play some role in this release. To determine whether the *Xenopus* melanotrope cells are unique with respect to the involvement of N-type channels in secretion, the effect of ω-conotoxin on melanotropes of other species should be examined.

We have recently shown that thapsigargin is effective in mobilizing intracellular Ca^{2+} in *Xenopus* melanotropes [37]. Thapsigargin specifically inhibits Ca^{2+}-ATPase of intracellular stores, without affecting calcium pumps in the plasma membrane [38,39,40]. In chicken gonadotrope cells thapsigargin treatment increases luteinizing hormone secretion [41]. We here show that this agent is ineffective in stimulating α-MSH secretion from *Xenopus* melanotropes. Apparently, mobilization of calcium from intracellular stores is not involved in stimulation of α-MSH secretion, even under conditions when calcium is rate-limiting. In contrast, the calcium ionophore A23187 significantly stimulated secretion under low Ca^{2+} conditions, most likely by causing an increased influx of extracellular Ca^{2+}. The absence of a clear effect of A23187 on spontaneous release under normal Ca^{2+} conditions indicates that the $[Ca^{2+}]_i$ is not rate-limiting under these conditions.

It is interesting to compare the electrophysiological data showing the presence of both nifedipine-sensitive and ω-conotoxin-sensitive Ca^{2+} channels with the secretory data that indicate that only ω-conotoxin-sensitive N-type channels are associated with the secretory process. Our results might reflect the existence of intracellular Ca^{2+} microdomains closely associated with a particular sub- population of Ca^{2+} channels with secretory granules. The idea of Ca^{2+} microdomains associated with secretion was first proposed for neurotransmitter release from the squid giant synapse [42,43] but may be more generally applicable to secretory processes [44]. In the case of *Xenopus* melanotropes, N-type channels could be closely associated with secretory granules and activity of these channels could create Ca^{2+} microdomains with an extremely high $[Ca^{2+}]_i$ to drive the secretory process. The L-type channels might not be associated with secretory granules and therefore, despite their presence on the melanotrope cell membrane, activation or inactivation of this channel type would have little or no effect on secretion.

It appears that, in general, secretion from most endocrine cells is supported by nifedipine-sensitive L-type channels, whereas release of neurotransmitters from nerve terminals is associated with the action of ω-conotoxin-sensitive channels. Therefore the findings of the present investigation, showing that spontaeous secretion of α-MSH from *Xenopus* melanotropes involves the action of ω-conotoxin-sensitive channels, suggests that these cells are a good model

system analysing the role of N-type channels in the regulation of secretory processes.

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SPONTANEOUS CALCIUM OSCILLATIONS IN *XENOPUS LAEVIS* MELANOTROPE CELLS ARE MEDIATED BY ω-CONOTOXIN-SENSITIVE CALCIUM CHANNELS

With Bruce G Jenks, Eric W Roubos and Peter H G M Willems

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The dynamics of intracellular Ca^{2+} signalling in single melanotrope cells of the pituitary gland of the amphibian *Xenopus laevis* have been studied by means of a digital imaging technique using the fluorescent dye Fura-2. When placed *in vitro*, the majority of the cells (77%) displayed spontaneous oscillatory changes in the free cytosolic Ca^{2+} concentration with a frequency of 1 ± 0.25 (S.D.) min^{-1}. The oscillations rapidly extincted when extracellular Ca^{2+} was reduced to nanomolar concentrations, revealing their complete dependence on Ca^{2+} influx. The fact that the Ca^{2+} oscillations were blocked by 1 μM ω-conotoxin, but not by nifedipine, at concentrations up to 50 μM, indicated that Ca^{2+} entered the cell via N-type rather than L-type voltage-operated Ca^{2+} channels. Thapsigargin, a putative inhibitor of intracellular Ca^{2+}-ATPase activity, elevated the baseline Ca^{2+} concentration but had no effect on the occurrence of the spontaneous oscillations. This suggests that intracellular Ca^{2+} pools are not involved in the mechanism underlying spontaneous Ca^{2+} oscillations. This is the first report showing spontaneous Ca^{2+} oscillations mediated by N-type Ca^{2+} channels in melanotrope cells.

The important role of intracellular Ca^{2+} ions in controlling cellular secretory activity is well established [1]. To date, increasing interest is being paid to the mechanisms that control the concentration of cytosolic free calcium ($[Ca^{2+}]_i$). The present study deals with this problem, with special attention to a cell type that transduces multiple neuronal inputs into a secretory signal: the melanotrope cell in the intermediate lobe of the pituitary gland [2-5]. Both in mammals and amphibians the activity of this cell type is regulated by stimulatory and inhibitory mechanisms effectuated by both classical neurotransmitters and neuropeptides. The products released from this cell are derived from the precursor protein proopiomelanocortin and include the melanotropic peptide, α-melanophore-stimulating hormone (α-MSH). The melanotrope cell shows spontaneous secretory activity, as shown in *in vitro* studies [2,3]. Its secretory activity is highly dependent on the presence of extracellular Ca^{2+} (Ca^{2+}_e) [6,7]. Melanotropes are electrically excitable cells spontaneously showing action potentials, in which Ca^{2+} enters the cell via voltage-operated Ca^{2+} channels [8-10]. In rat melanotropes it has been shown that these channels show similarities to L-, N- and T-type voltage-operated Ca^{2+} channels [11].

In many excitable cells an interesting phenomenon has been observed with respect to intracellular Ca^{2+} dynamics, viz. temporal concentration changes in the form of oscillations [12-18]. These oscillations can occur spontaneously or after receptor activation. Although the physiological relevance of the spontaneous oscillations is poorly understood, some insight into their generation has been obtained. First, for some endocrine cells (e.g. lactotropes, somatotropes and corticotropes) it has been shown that they are generated by an influx of Ca^{2+}_e. In

the rat endocrine chromaffin cell, on the other hand, Ca^{2+} oscillations originate from intracellular stores that release Ca^{2+} by opening of Ca^{2+}- activated Ca^{2+} channels in the endoplasmic reticulum ('Ca^{2+}-induced Ca^{2+} release'; CICR [19]).

In the present study the dynamics of changes in intracellular Ca^{2+} concentration in the endocrine melanotrope cell of the amphibian *Xenopus laevis* have been investigated. The melanotrope cell of *Xenopus* is very suitable to examine secretory control mechanisms because its secretion can be physiologically activated by adapting the animal to a black background [20]. To monitor the dynamics of $[Ca^{2+}]_i$, single melanotropes were studied using the fluorescent Ca^{2+} indicator Fura-2 and a digital imaging technique. The majority of the cells display spontaneous Ca^{2+} oscillations, which completely depend on Ca^{2+}_e. It appears that, remarkably for pituitary secretory cells, the oscillations of *Xenopus* melanotropes are generated by ω-conotoxin-sensitive N-type Ca^{2+} channels.

MATERIALS AND METHODS

Animals

Young-adult *Xenopus laevis* were taken from laboratory stock and adapted to a black background for three weeks under continuous illumination at 22 °C. The animals were fed weekly with beef heart.

Preparation of isolated cells

Isolation of melanotrope cells was performed as described previously [21,22] with minor changes. In short, after perfusing the animal with *Xenopus* Ringer's solution containing 112 mM NaCl, 15 mM Ultral-HEPES (Calbiochem, La Jolla CA, USA; pH 7.4), 2 mM KCl and 2 mM $CaCl_2$, to remove blood cells, lobes were dissected and then incubated for 45 min in Ringer's solution without $CaCl_2$, to which 0.25 % (w/v) trypsin (Gibco, Renfrewshore, UK) had been added. Cells were subsequently dispersed in Leibovitz's L15 medium to which 10 % fetal calf serum (Gibco) was added, by gentle trituration of the lobes with a siliconized Pasteur's pipette. The medium was adjusted to *Xenopus* osmolality (L15:Milli Q = 2:1). After washing, the cells were plated on cover slides coated with poly-L-lysine (Sigma, St. Louis, MO, USA; Mw > 300 kD), at a density of about 10,000/slide, and cultured for 3 days, at 22 °C.

Ca^{2+} measurements

Cells were loaded with 2 μM Fura-2/ AM (Molecular Probes, Eugene, OR, USA) in Ringer's solution containing 1 μM pluronic F127 [23] (Molecular Probes), for 20 min at 22 °C. In preliminary experiments with *Xenopus* melanotropes it was found that pluronic F127 does not affect the oscillations but greatly enhances the trapping of the dye into the cytosol [see also ref. 23]. Therefore, this substance was included in our standard protocol. After loading, cells were washed with Ringer's solution in a Leiden perifusion chamber [24] (volume

800 µl) at a rate of 1 ml/min, for 25 min During this wash unattached cells were sucked off and attached cells were allowed to equilibrate The chamber was placed on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan) The light from a 100 W xenon lamp was directed through a quartz neutral density filter (ND 2, Ealing Electro-Optics, Holliston, MA, USA) to reduce bleaching of the intracellularly trapped fluorochrome The excitation bandpass filters, mounted in a motor-driven rotating wheel, had transmission maxima at 340 and 380 nm (± 12 nm) (Ealing Electro-Optics) The fluorescence emission ratio at 492 nm was used as a measure of $[Ca^{2+}]_i$ after excitation at 340 and 380 nm [25] An epifluorescent 40x magnification oil immersion objective was used Dynamic video imaging was carried out using the MagiCal hardware and TARDIS software of Joyce Loebl (Dukesway, Team Valley, Gateshead, Tyne & Wear, UK) as described in detail by Neylon et al [26] The interframe interval between the ratio frames was 6 4 sec with a maximal sampling time of 32 min

In a typical experiment the fluorescence intensity decreased by 10 % in 30 min Several processes may be responsible for the loss of the fluorescence intensity including efflux of the probe to the medium To test this possibility, probenicid, an inhibitor of organic anion transporter activity [27], was added to the superfusion medium In the presence of probenicid (3 mM), the decrease in fluorescence was reduced to 5% in 30 min (N=26) However, probenicid markedly broadened the Ca^{2+} transients (Fig 1B) and was therefore not used in our standard procedure It is unclear how probenicid exerts its effect but it may be that it increases the gating probabilities of Ca^{2+} channels or inhibits Ca^{2+} extrusion mechanisms

Chemicals

Test substances were added to the bath solution via a perifusion pump Nifedipine and [ethylenebis(oxyethylenenitrilo)] tetraacetic acid (EGTA) were obtained from Sigma, ω-conotoxin GVIA from Peninsula Labs Inc (Belmont, CA, USA) and thapsigargin and probenicid from Molecular Probes In order to obtain a constant osmolality in all the experiments, the concentration of NaCl was adjusted in the experiments where EGTA was added

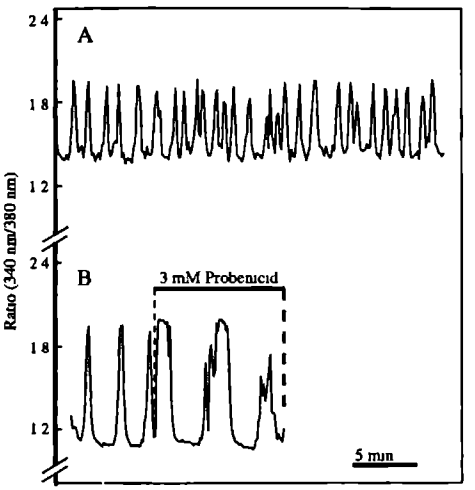

Figure 1: Spontaneous Ca^{2+} oscillations in single melanotrope cells of *Xenopus laevis* Melanotropes, isolated by enzymatic digestion and subsequently cultured for 3 days, were loaded with 2 µM Fura-2/AM in the presence of 1 µM pluronic F127 for 20 min at 20 °C After a wash of 20 min, to remove non-hydrolysed dye, fluorescence measurements were started The fluorescence emission ratio at 492 nm is shown as a measure of $[Ca^{2+}]_i$ after excitation at 340 and 380 nm **A**, spontaneous Ca^{2+} oscillations in a *Xenopus* melanotrope **B**, the effect of 3 mM probenicid, added to the bath solution by perifusion

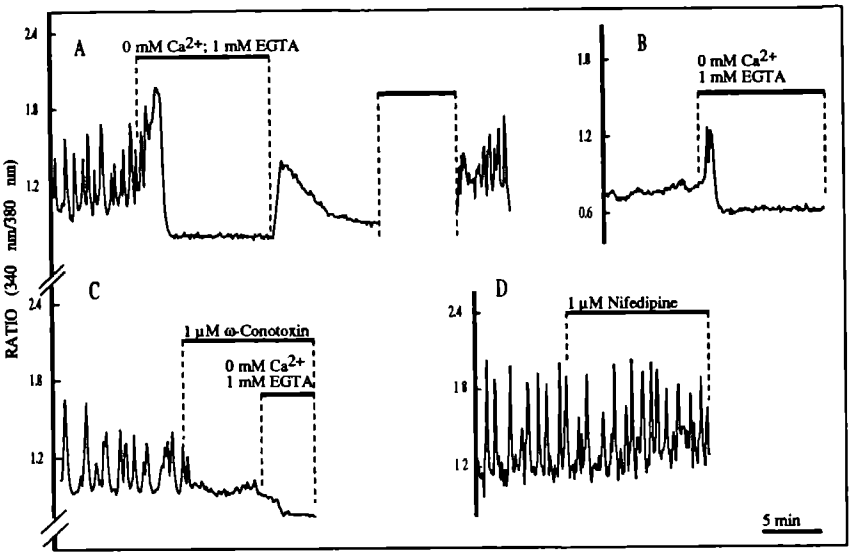

Figure 2: The effects of low $[Ca^{2+}]_e$, ω-conotoxin and nifedipine on spontaneous Ca^{2+} oscillations and baseline $[Ca^{2+}]_i$. Loading of melanotropes with Fura-2 and fluorescence measurements were performed as described in the caption of Fig. 1. **A**, effect of reduction of $[Ca^{2+}]_e$ to 10 nM, by omission of Ca^{2+}_e and inclusion of 1 mM EGTA, on a spontaneously oscillating melanotrope. **B**, a similar experiment using a cell that does not display spontaneous Ca^{2+} oscillations. **C**, effect of addition of 1 µM ω-conotoxin followed by the reduction of $[Ca^{2+}]_e$. **D**, lack of effect of 1 µM nifedipine on the same cell as shown in C, that had recovered from ω-conotoxin and EGTA treatment by an extensive wash with normal Ringer's solution for 20 min.

RESULTS

Demonstration of spontaneous Ca^{2+} oscillations

Using digital imaging microscopy of Fura-2-loaded single *Xenopus* melanotropes, spontaneous oscillatory changes in free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) were demonstrated. The oscillations consisted of series of distinct Ca^{2+} transients originating from a baseline Ca^{2+}_i level (Fig. 1A). In each experiment, oscillations in up to 20 individual melanotrope cells could be monitored simultaneously. Spontaneous Ca^{2+} oscillations occurred in 77% of the cells (240 out of 312 cells). The averaged frequency of the spontaneous oscillations was 1 ± 0.25 min^{-1} (N=240) but appeared to be highly variable among individual cells, ranging from 0.3 to 4 min^{-1}. However, for any given cell, the oscillation frequency proved to be markedly constant.

Involvement of Ca^{2+}_e in oscillations

Reduction of the $[Ca^{2+}]_e$ from 2 mM to 10 nM by the omission of Ca^{2+}_e and the addition of EGTA (1 mM) caused an instantaneous transitory and relatively large increase in fluorescence emission ratio followed by a complete block of spontaneous oscillations and a decrease of the baseline value by 25 ± 5 % (Fig. 2A, N=73). Return to normal Ringer's solution immediately restored the $[Ca^{2+}]_i$ to near baseline levels. Spontaneous oscillations reappeared only after 15 to 20 min, indicating that the cells remained viable. In non-oscillating cells perifusion with 10 nM Ca^{2+}-medium also led to an instantaneous large increase in fluorescence emission ratio, which was followed by a decrease to a value below the baseline level (Fig. 2B, N=22).

Involvement of voltage-operated Ca^{2+} channels in oscillations

In order to investigate the mechanism that is involved in Ca^{2+} entry into the melanotrope, the Ca^{2+} channel inhibitors $CoCl_2$ (12), nifedipine [28,30] and

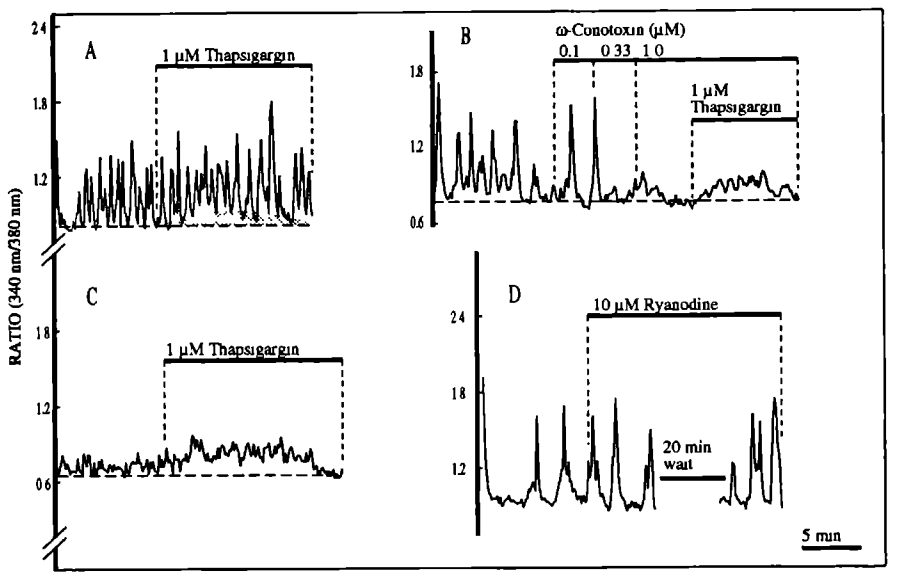

Figure 3: Effects of thapsigargin, ω-conotoxin and ryanodine on spontaneous Ca^{2+} oscillations and baseline $[Ca^{2+}]_i$. Loading of melanotropes with Fura-2 and fluorescence measurements were performed as described in the caption of Fig. 1. **A**, effect of 1 µM thapsigargin on a spontaneously oscillating melanotrope; the hatching indicates the period of time that the fluorescence emission ratio is slightly but consistently elevated by the action of thapsigargin. **B**, effect of thapsigargin in the presence of ω-conotoxin on a spontaneously oscillating melanotrope. **C**, experiment similar to that shown in A but conducted on a non-oscillating cell. **D**, effect of 10 µM ryanodine on a spontaneously oscillating melanotrope. During the 20 min waiting period the cell was continuously perifused with ryanodine-containing Ringer's solution.

ω-conotoxin [31] were studied. Adding $CoCl_2$ (1 mM) effectively inhibited spontaneous Ca^{2+} oscillations in all cells tested, without affecting baseline $[Ca^{2+}]_i$ (N=21, data not shown). Nifedipine had no effect on spontaneous oscillations when used at concentrations of 1 μM (Fig. 2D, N=35), 10 μM (N=35) or 50 μM (N= 14) ω-Conotoxin (1 μM) on the other hand, rapidly and completely inhibited spontaneous Ca^{2+} oscillations in all cells studied without affecting baseline $[Ca^{2+}]_i$ (Fig. 2C, N=60). At a concentration of 0.33 μM the inhibitor reduced the frequency of the Ca^{2+} oscillations without blocking the oscillations in most cells studied (N=17). Removal of ω-conotoxin resulted in the reappearance of the spontaneous oscillations, but only after an extensive wash of at least 20 min. In the 10 nM Ca^{2+}-medium the ω-conotoxin-treated cells showed a decrease in baseline $[Ca^{2+}]_i$ (Fig. 2C). However, the relatively large increase in fluorescence emission ratio observed in control cells (see Fig. 2A) did not occur in the presence of ω-conotoxin. This toxin had no effect on non-oscillating cells (data not shown).

Dependence on intracellular Ca^{2+} stores

Thapsigargin, an inhibitor of internal Ca^{2+}-ATPase activity [32], was used to study a possible role of internal Ca^{2+} stores in spontaneous Ca^{2+} oscillations. At 1 μM, thapsigargin did not affect the spontaneous oscillations (Fig. 3A, N=32). Under conditions where spontaneous Ca^{2+} oscillations were blocked by ω-conotoxin, thapsigargin did not induce Ca^{2+} oscillations (Fig. 3B, N=17).

Both in the absence and presence of ω-conotoxin, thapsigargin slightly but consistently elevated the baseline fluorescence emission ratio (Fig. 3A,B). In non-oscillating cells thapsigargin also evoked a transient elevation of baseline $[Ca^{2+}]_i$ (Fig. 3C, N=12). The effect of thapsigargin lasted for approximately 15 min.

Ryanodine, a blocker of CICR [33-35], did not block the spontaneous oscillations when applied at concentrations of 5, 10 (Fig. 3D, N=35), 50 or 500 μM, for periods up to 30 min.

DISCUSSION

Spontaneous Ca^{2+} oscillations have been observed in various pituitary cell types. Generally, for any given cell type, only a small percentage of the cells has been reported to display spontaneous oscillations [12-18]. We now demonstrate that, in contrast to other pituitary cells, the vast majority of *Xenopus* melanotropes exhibit spontaneous oscillations. The occurrence of spontaneous Ca^{2+}

oscillations may be a characteristic feature of *Xenopus* melanotropes, because spontaneous oscillations could not be demonstrated in rat melanotropes [36,37].

Melanotropes show spontaneous secretory activity. This suggests that spontaneous Ca^{2+} oscillations are somehow related to the process of exocytosis. Since the spontaneous *in vitro* secretory activity of *Xenopus* melanotropes strongly depends on the presence of Ca^{2+}_e [6] we tested the dependence of the oscillations on Ca^{2+}_e. The spontaneous oscillations appeared to be effectively and reversibly blocked by reducing $[Ca^{2+}]_e$ to 10 nM. From this observation it is concluded that Ca^{2+}_e is crucial for the generation of spontaneous Ca^{2+} oscillations in *Xenopus* melanotropes. Intracellular stores, if involved in the process, are unable to maintain the oscillations for sustained periods of time. Apparently, the dependence of the spontaneous oscillations on Ca^{2+}_e is a general feature of pituitary cells, because it has also been demonstrated for a number of anterior lobe endocrine cell types [12-18].

Cobalt effectively inhibited the spontaneous Ca^{2+} oscillations, indicating the involvement of Ca^{2+} channels in oscillatory activity [12]. In order to investigate which type of channel plays a role in Ca^{2+} entry, the effects of specific blockers of voltage-operated Ca^{2+}-channels were investigated. Nifedipine, which specifically binds to L-type channels [28-30], had no effect on spontaneous Ca^{2+} oscillations when added at concentrations up to 50 μM, indicating that L-type channels are not involved in spontaneous Ca^{2+} oscillations. Concentrations of 1 to 10 μM nifedipine have been demonstrated to be sufficient to block oscillations in $[Ca^{2+}]_i$ in other pituitary cell types [for references, see 12,15]. The possibility of species differences in sensitivity to nifedipine seems to be excluded because we were able to block Ca^{2+} current activity in *Xenopus* melanotropes in patch clamp studies. On the other hand, 1 μM ω-conotoxin was effective in blocking spontaneous oscillations of *Xenopus* melanotropes, suggesting that ω-conotoxin-sensitive N-type channels are involved in the generation of these spontaneous Ca^{2+} oscillations. The relative high dose of ω-conotoxin needed to completely block the oscillations in amphibian tissue, compared to the effective concentration to block N-type channels in mammalian studies, might reflect species differences and/or the use of reduced temperatures. However, it should be noted that in mammalian cell preparations the EC_{50} of ω-conotoxin with respect to its biological action has been found to be higher than the Kd for its action as a Ca^{2+}-channel blocker [31]. In the present study, lower concentrations of ω-conotoxin were found to reduce the frequency of the Ca^{2+} oscillations. Possibly, in this situation the Ca^{2+} influx rate is reduced so that the time to reach the threshold value for the induction of a further Ca^{2+}-influx is prolonged [15].

It is generally accepted that ω-conotoxin has irreversible binding properties [30,31]. We found that the inhibitory effect of ω-conotoxin could only be abolished by extensive washing for at least 20 min. A possible explanation for the slow recovery might be the recruitment of new channels. Such a recruitment has recently been reported for voltage-operated Ca^{2+} channels [38,39] and Na^{+} channels [40].

Reduction of the medium [Ca^{2+}] to 10 nM evoked an instantaneous Ca^{2+} transient. Such a Ca^{2+} transient has been shown for rat chromaffin cells [19]. In contrast to *Xenopus* melanotropes, in chromaffin cells the relatively large increase in $[Ca^{2+}]_i$ marks the end of a period of oscillating activity lasting 1 to 5 min from the onset of the Ca^{2+}-free conditions. The ability of these cells to maintain oscillating activity under such conditions suggests the involvement of intracellular Ca^{2+} stores. The capability of ω-conotoxin to eliminate the large transient in *Xenopus* melanotropes indicates the involvement of N-type Ca^{2+} channels in producing this transient. This is in agreement with electrophysiological studies that have shown that opening of N-type Ca^{2+} channels is Ca^{2+}-dependent with a reduction of $[Ca^{2+}]_e$ leading to an increase in the gating properties of these channels [41].

In contrast to Ca^{2+}-free medium, ω-conotoxin did not reduce the baseline $[Ca^{2+}]_i$ thus indicating that N-type channels are not involved in maintaining baseline [Ca^{2+}] in *Xenopus* melanotropes. In other pituitary cell types, in which Ca^{2+} oscillations depend on L-type channels, blocking of the L-type channels not only inhibits Ca^{2+} oscillations but also lowers the $[Ca^{2+}]_i$ to levels similar to those observed in the absence of Ca^{2+}_e [for references, see 12,15]. Our results showing that nifedipine had no effect on $[Ca^{2+}]_i$ indicates that also the L-type channel has no important role in maintaining baseline $[Ca^{2+}]_i$ in *Xenopus* melanotropes. Therefore, Ca^{2+} transporters other than N-type and L-type channels must be involved in maintaining the baseline $[Ca^{2+}]_i$. Interestingly, in melanotropes of the amphibian *Rana ridibunda* the presence of a non-selective cation channel has been demonstrated [42], leaving the possibility that in *Xenopus* such a channel is involved in the maintenance of the baseline $[Ca^{2+}]_i$.

Recently, it has been reported that thapsigargin can induce oscillations in rat parotid cells [33,43]. In addition, it was shown that prolonged stimulation with thapsigargin leads to the depletion of internal Ca^{2+} stores [33,43]. The present observation that oscillations can not be blocked by thapsigargin suggests that thapsigargin-sensitive Ca^{2+} stores are not involved in spontaneous Ca^{2+} oscillations in *Xenopus* melanotropes. Both in ω-conotoxin-inhibited spontaneously active cells and in silent cells, thapsigargin induced a relatively small transient increase in fluorescence emission ratio, indicating that a thapsigargin-sensitive

intracellular Ca^{2+} store is present in these melanotropes. Apparently, an increase in cytosolic Ca^{2+} alone, as observed in thapsigargin-treated silent cells, is insufficient to trigger CICR. In mammalian skeletal and cardiac muscle, ryanodine inhibits CICR [33-35]. While amphibians clearly have ryanodine receptors structuraly related to those of mammals [44], ryanodine was ineffective in blocking the oscillations in *Xenopus* melanotropes. This observation supports the conclusion that these oscillations depend solely on Ca^{2+}_e. Another tool to study CICR is caffeine [19,34,35,43]. However, we have found that caffeine affects the spontaneous oscillations of *Xenopus* melanotropes most probably by increasing the intracellular cyclic-AMP concentrations, due to its inhibitory action on the cyclic nucleotide phosphodiesterase[1]. Therefore, caffeine can not be used to study the involvement of CICR in spontaneous oscillations in *Xenopus* melanotropes.

Based on the observations presented in this paper, spontaneous Ca^{2+} oscillations in *Xenopus* melanotropes can be explained by a periodic opening of N-type voltage-operated Ca^{2+} channels. Opening of these channels can take place as a consequence of spontaneous membrane depolarizations [45]. A thapsigargin-sensitive internal store is not directly involved in either the generation or the maintenance of spontaneous oscillations. However, in analogy with the situation in non-excitable secretory cells, such a store might be sensitive to inositol 1,4,5-trisphosphate produced upon receptor activation [46]. In order to establish a possible relationship between spontaneous Ca^{2+} oscillations and secretion we are currently examining the effects of neurochemical messengers, with well known stimulatory and inhibitory action on MSH secretion, on the spontaneous Ca^{2+} oscillations of *Xenopus* melanotropes.

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ACTION OF STIMULATORY AND INHIBITORY α-MSH SECRETAGOGUES ON SPONTANEOUS CALCIUM OSCILLATIONS IN MELANOTROPE CELLS OF *XENOPUS LAEVIS*

With Bruce G Jenks, Peter H G M Willems and Eric W Roubos

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The secretion of α-melanophore stimulating hormone (α-MSH) from melanotrope cells in the pituitary gland of *Xenopus laevis* is regulated by various neural factors, both classical neurotransmitters and neuropeptides. The majority of these cells (80%) display spontaneous Ca^{2+} oscillations. In order to gain a better understanding of the external regulation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in the melanotrope cell, we have examined the action of well known α-MSH secretagogues on the Ca^{2+} oscillations. It is shown that all secretagogues tested also control the oscillatory state of *Xenopus* melanotropes, viz. the secreto-inhibitors dopamine, isoguvacine ($GABA_A$-agonist), baclofen ($GABA_B$-agonist) and neuropeptide Y evoked a rapid quenching of the spontaneous Ca^{2+} oscillations, whereas the secreto-stimulant sauvagine, an amphibian peptide related to corticotropin releasing hormone, induced oscillatory activity in non-oscillating cells. Argumentation is given for the idea that the regulation of Ca^{2+} oscillations is a focal point in the regulation of secretory activity of melanotrope cells. There was considerable heterogeneity among melanotrope cells in the threshold of their Ca^{2+} response to secretagogue treatment. This heterogeneity may be the basis for melanotrope cell recruitment observed during physiological adaptations of the animal to the light intensity of its background.

The South African clawed toad *Xenopus laevis* is capable of adapting its skin color to the gray level of the background. Melanotrope cells of the pituitary pars intermedia play an important role in this process. In animals kept on a black background, these cells release α-melanophore stimulating hormone (α-MSH) which causes darkening of the skin by inducing dispersion of the pigment melanin in the dermal melanophores [10]. Using a fluorescent Ca^{2+} probe and video imaging microscopy, we previously demonstrated that 80% of single *Xenopus* melanotropes kept *in vitro* display spontaneous, repetitive, transient elevations of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and that these oscillations depend completely on extracellular Ca^{2+} (Ca^{2+}_e) entering the cell via ω-conotoxin-sensitive N-type Ca^{2+} channels [22]. As a logical sequel to this study, we have now examined if the Ca^{2+} oscillations are under external control by studying the effects of various neural messengers known to influence the secretory activity of *Xenopus* melanotropes. In order to gain a better understanding of the possible relationship of Ca^{2+} oscillations and secretion [14, 24], special attention has been paid to the effects of these neural messengers on the induction or inhibition of these oscillations and on the level of cytosolic $[Ca^{2+}]$ (baseline $[Ca^{2+}]_i$). In rat gonadotropes and corticotropes that also exhibit spontaneous Ca^{2+} oscillations that depend on entry of Ca^{2+}_e [7,8], secretion does not depend on Ca^{2+}_e. For rat gonadotropes it has been proposed that the baseline $[Ca^{2+}]_i$ is related to secretion [16].

Melanotrope cells of *Xenopus* display sustained spontaneous secretory activity *in vitro* [10]. This activity is inhibited by dopamine, neuropeptide Y (NPY) and γ-aminobutyric acid (GABA) [27,29,31]. Since dopamine acts

Table 1 Summary of the effects of secretagogues on changes in $[Ca^{2+}]_i$ in non-oscillating and spontaneously oscillating melanotrope cells of *Xenopus laevis*

TREATMENT(S)	OSCILLATING CELLS		NON-OSCILLATING CELLS
	OSCILLATIONS	BASELINE	
Dopamine	inhibition	none (or ↓)	none or ↓(baseline)
Dopamine + sulpiride	none	none	not determined
Isoguvacine (low)	frequency ↑	none	transient
Isoguvacine (high)	inhibition	↑	transient
Isoguvacine (low) + picrotoxin	none	none	none
Isoguvacine (high) + picrotoxin	none	transient ↑	transient
Isoguvacine (low) + bicuculline	none	none	not determined
Isoguvacine (high) + bicuculline	none	none	not determined
Baclofen	inhibition	none (or ↓)	none
Baclofen + phaclofen	none	none	not determined
Neuropeptide Y	inhibition	none	none
TRH	none	transient ↑	none
Sauvagine	frequency ↑	none (or ↑)	none or oscillations

through a D_2 receptor and GABA through $GABA_A$ and $GABA_B$ receptors, specific agonists and antagonists have been included in the present study of Ca^{2+} dynamics. Furthermore, the neuropeptides sauvagine and thyrotropin-releasing hormone (TRH) have been included. Sauvagine, an amphibian peptide related to mammalian corticotropin releasing hormone, stimulates α-MSH secretion [28]. TRH is also known to stimulate secretion from *Xenopus* melanotropes, but only in lobes derived from white-adapted animals [30]. The experiments were performed with Fura-2-loaded single melanotropes in superfusion, using video imaging microscopy.

MATERIALS AND METHODS

Animals

Young-adult *Xenopus laevis* were taken from laboratory stock and adapted to a black background for three weeks under continuous illumination, at 22 °C The animals were fed weekly with beef heart

Preparation of single cells

Isolation of melanotrope cells was performed as described previously [2,5], with minor changes In short, animals were anaesthetized for 10 to 20 min in a solution containing 1 g/l MS222 (Sigma, St Louis, MO, USA) and 1 5 g/l $NaHCO_3$ (pH 6 8) After perfusing the animal with *Xenopus* Ringer's solution, containing 112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$ and 15 mM Ultral-HEPES (Calbiochem, La Jolla CA, USA, pH 7 4) to remove blood cells,

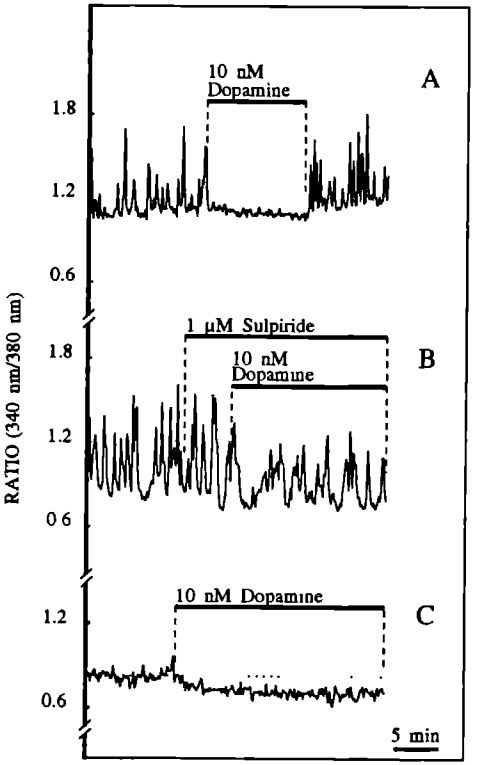

Figure 1. The effects of dopamine on oscillating and non-oscillating cells.

Melanotropes, isolated by enzymatic digestion and subsequently cultured for 3 days, were loaded with 2 µM Fura-2/AM in the presence of 1 µM pluronic F127 for 20 min at 20 °C. After a wash of 20 min to remove non-hydrolysed dye, fluorescence measurements were started. The fluorescence emission ratio at 492 nm is shown as a measure of $[Ca^{2+}]_i$ after excitation at 340 and 380 nm. **A**, The minimum effective concentration of dopamine to completely block spontaneous oscillations was 10 nM. **B**, When 10 nM dopamine was given in the presence of 1 µM sulpiride its action was reversed. **C**, In 20 % of the non-oscillating cells dopamine induced a decrease in the baseline $[Ca^{2+}]_i$.

neurointermediate pituitary lobes were dissected and incubated for 45 min in Ringer's solution without $CaCl_2$ to which 0.25 % (w/v) trypsin (Gibco, Renfrewshore, UK) was added. Cells were subsequently dispersed in Leibovitz's L15 medium, containing 10 % fetal calf serum (Gibco), by gentle trituration of the lobes with a siliconized Pasteur's pipette. The medium had been adjusted to *Xenopus* blood osmolality (L15 : ultrapure water = 2.1). After washing, the cells were plated on glass cover slips coated with poly-L-lysine (Sigma, Mw > 300 kD) at a density of about 10,000 cells/slip, and cultured for 3 days at 22 °C. The cells were readily identified on the basis of their characteristic round shape.

$[Ca^{2+}]_i$ measurements

Measurements of intracellular Ca^{2+} were performed as described previously [22] In short, cells were loaded with 2 µM Fura-2/AM (Molecular Probes, Eugene, OR, USA) in Ringer's solution containing 1 µM pluronic F127 [21] (Molecular Probes), for 20 min, at 22 °C. Then cells were washed with Ringer's solution in a Leiden perifusion chamber [9] (volume 800 µl) at a flow rate of 1 ml/min, for 25 min, to remove non-hydrolysed dye During washing unattached cells were sucked off and attached cells were allowed to equilibrate The chamber was placed on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan). The light from a 100 W xenon lamp was directed through a quartz neutral density filter (ND 2, Ealing Electro-Optics, Holliston, MA, USA) to reduce bleaching of the intracellularly trapped dye. The excitation bandpass filters (Ealing Electro-Optics), mounted in a motor-driven rotating wheel, had transmission maxima at 340 and 380 nm (± 12 nm). The fluorescence emission ratio at 492 nm was used as a measure of $[Ca^{2+}]_i$ after excitation at 340 and 380 nm [20]. An

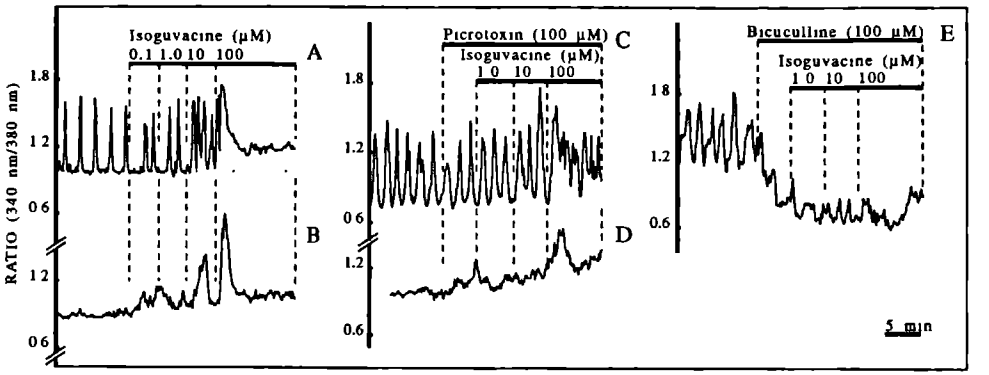

Figure 2. The effects of $GABA_A$ receptor activation by isoguvacine on oscillating and non-oscillating cells **A** and **B**, Dose-response relationship for isoguvacine on oscillating and non-oscillating cells, respectively. **C** and **D**, The effects of the chloride channel blocker picrotoxin on the biphasic effect of isoguvacine in oscillating and non-oscillating cells. **E**, The effect of bicuculline on isoguvacine-induced changes in oscillating cells. The decrease in the baseline fluorescence emission ratio under bicuculline is due to the autofluorescent properties of this pharmacon when excited at 380 nm (Methodology: see Fig. 1).

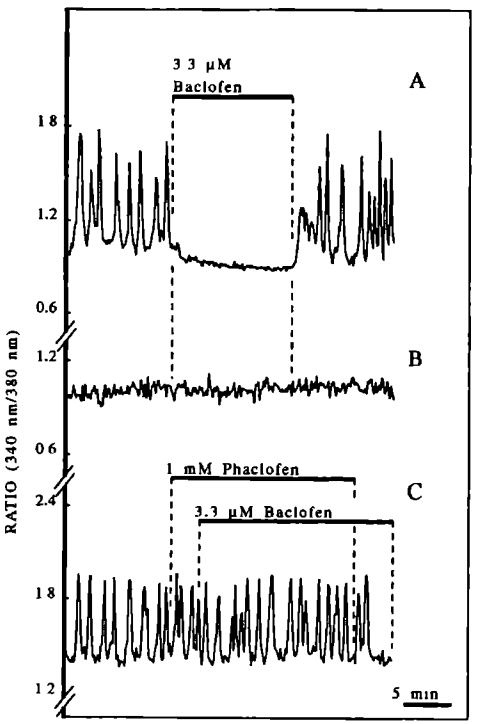

Figure 3. The effects of $GABA_B$ receptor activation by baclofen on oscillating and non-oscillating cells. **A**, baclofen (3.3 μM) reversibly blocks spontaneous oscillations. **B**, baclofen has no effect on non-oscillating cells. C, The inhibition by baclofen is effectively blocked by 1 mM phaclofen. (Methodology: see Fig 1).

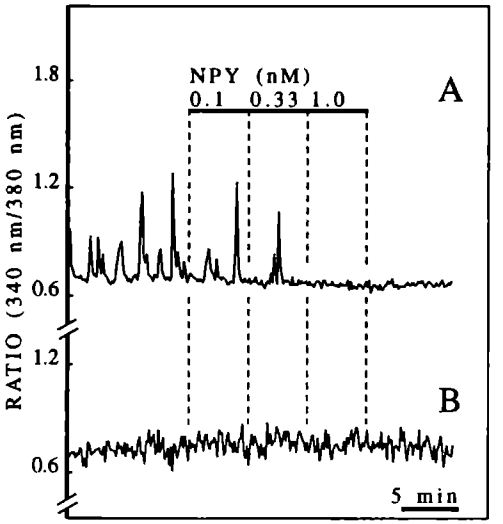

Figure 4. Effects of NPY on oscillating and non-oscillating cells. **A**, Neuropeptide Y potently blocked spontaneous Ca^{2+} oscillations at 1 nM. At 0.33 nM oscillations were blocked in 40 % of the cells. The effect of NPY was not easily reversible. **B**, NPY had no effect on non-oscillating cells. (Methodology: see Fig. 1).

epifluorescent 40x magnification oil immersion objective was used. Dynamic video imaging was carried out using the MagiCal hardware and TARDIS software of Joyce Loebl (Dukesway, Team Valley, Gateshead, Tyne & Wear, UK) as described in detail by Neylon et al. [19]. The interframe interval between the ratio frames was 6.4 s with a maximal sampling time of 32 min.

Pharmaca

Neural factors, agonists and antagonists were added to the bathing medium via the perifusion pump. NPY, sauvagine and TRH were obtained from Bachem Feinchemikalien AG (Basel, Switzerland); sulpiride, baclofen, phaclofen and isoguvacine were from Research Biochemicals Inc. (Natick, MA, USA); dopamine and bicuculline were from Sigma.

RESULTS

Approximately 80 % of the melanotropes displayed spontaneous Ca^{2+} oscillations, rising from baseline $[Ca^{2+}]_i$. Although the frequency of the oscillations and baseline ratio values were found to be highly variable among individual oscillating cells, they proved to remain markedly constant for any given cell for periods up to 1 hour. Preliminary experiments had revealed that individual cells respond with different sensitivities to a particular secretagogue. Therefore, the dose-dependency of the effect of each secretagogue was examined using the same cell. The effects of secretagogues included both inhibition or induction (in non-oscillating cells) of Ca^{2+} oscillations and changes in oscillation frequency. These effects are summarized in Table 1 and described in further detail below.

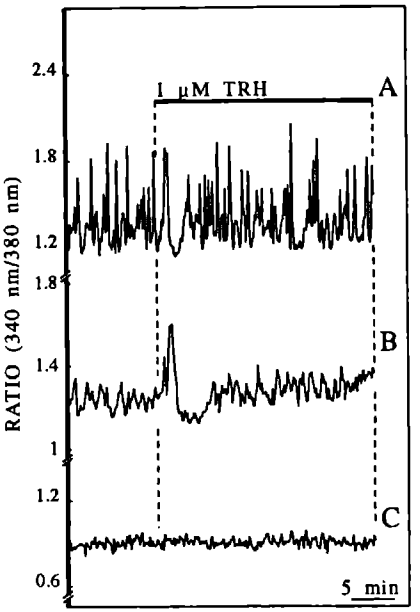

Figure 5. Effects of TRH on oscillating and non-oscillating cells. **A**, TRH caused a short-term effect on oscillating cells, consisting of a 2 min lasting transient spike followed by a period of 2 to 3 min during which all oscillating activity was absent. After this period oscillations reappeared. **B**, averaging the signals of all oscillating cells shows the TRH-induced transient more clearly. **C**, TRH had no effect on non-oscillating cells. (Methodology: see Fig. 1).

Dopamine

Dopamine completely blocked spontaneous oscillations in all cells studied (N=38) at a minimal effective concentration of 10 nM (Fig. 1A). In 26 % of the oscillating cells a subsequent gradual decrease in baseline $[Ca^{2+}]_i$ was observed. Removing dopamine from the superfusion medium resulted in a rapid reappearance of the oscillations within 30 seconds. At 1 nM, dopamine did not inhibit the oscillations. The inhibitory action of 10 nM dopamine on oscillating cells was blocked by 1 µM of the D_2-antagonist sulpiride (Fig. 1B, N=9). Sulpiride itself had no effect on oscillating cells. In non-oscillating cells (N=10) dopamine (10 nM) either had no effect (80 % of the cells) or reduced the baseline $[Ca^{2+}]_i$ (Fig. 1C).

$GABA_A$

The $GABA_A$ agonist isoguvacine had a dual effect on spontaneous Ca^{2+} oscillations. When applied at a concentration of 10 µM it increased the frequency of the oscillations in all cells studied (Fig. 2A, N=9). In 2 out of 9 cells this increase was already observed at 1 µM. However, when applied at a high dose (100 µM) isoguvacine evoked a marked Ca^{2+} transient followed by inhibition of the spontaneous oscillations (Fig. 2A). This inhibition was paralleled by an increase in baseline $[Ca^{2+}]_i$ (Fig. 2A). This response was found in all oscillating cells. In 2 out of 4 non-oscillating cells, isoguvacine caused single, large

transients, the amplitude of which increased in a dose-dependent manner upon stepwise increasing the isoguvacine concentration (Fig. 2B). The post-transient baseline $[Ca^{2+}]_i$ was further increased by stepwise elevation of the isoguvacine concentration. The $GABA_A$ receptor antagonist bicuculline (100 µM) blocked both the stimulatory and inhibitory action of isoguvacine in oscillating cells (Fig. 2E, N=6). In addition, the large Ca^{2+} transient normally observed upon stimulation with 100 µM isoguvacine was not observed in the presence of bicuculline. The antagonist alone appeared to decrease the baseline ratio fluorescence signal and the amplitude of the oscillations, but this was due to the autofluorescent properties of bicuculline at 380 nm (data not shown). Both the stimulatory and the inhibitory effect of isoguvacine on the Ca^{2+} oscillations were abolished by adding the chloride channel blocker picrotoxin (100 µM; Fig. 2C, N=7). However, picrotoxin did not block the pronounced Ca^{2+}-transient evoked by 100 µM isoguvacine in oscillating and non-oscillating (N=3) cells (Fig. 2C, D). Picrotoxin alone had no effect on oscillating or non-oscillating cells.

$GABA_B$

The $GABA_B$ agonist baclofen completely blocked spontaneous oscillations in 6 out of 14 melanotropes when applied at a concentration of 0.1 µM. To block oscillations in all cells, a concentration of 3.3 µM baclofen was required (Fig. 3A, N=14). In 20 % of the cells a subsequent gradual decrease in baseline $[Ca^{2+}]_i$ was observed. Removal of baclofen resulted in an immediate reappearance of the oscillations. The inhibitory effect of baclofen could completely and reversibly be blocked by the $GABA_B$-antagonist phaclofen (1 mM; Fig. 3C, N=6). Baclofen had no effect on non-oscillating cells (Fig. 3B, N=4).

Neuropeptide Y

NPY inhibited the oscillations in all cells at a concentration of 1 nM (Fig. 4A, N=20). The cells displayed different sensitivities to NPY because at 0.33 nM, 40 % of the cells had become silent whereas the rest of the cells still displayed Ca^{2+} oscillations. The inhibitory effect of NPY was not easily reversible; even after an extensive wash of 30 min the oscillations did not fully reappear. NPY did not affect the baseline $[Ca^{2+}]_i$ of oscillating cells and had no effect on non-oscillating cells (Fig. 4B, N=5).

Thyrotropin Releasing Hormone

In oscillating cells TRH caused a Ca^{2+} transient of prolonged duration (1 to 2 min) when applied at a concentration of 0.1 or 1 µM (N=19) after which oscillations stopped for about 2 to 3 min but then reappeared (Fig. 5A). To demonstrate the effect of TRH in another way, the signals of all individual oscillating cells were averaged, making the individual oscillations indistinguishable. In this way the TRH-evoked transient became very

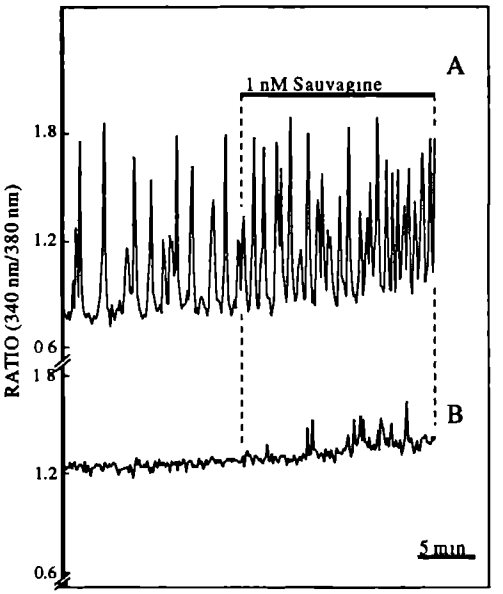

Figure 6. Effects of sauvagine on oscillating and non-oscillating cells. **A**, Sauvagine (1 nM) increased the frequency of spontaneous Ca^{2+} oscillations. **B**, Sauvagine induced Ca^{2+} oscillations in 33% of the non-oscillating cells. (Methodology: see Fig. 1).

conspicuous (Fig. 5B). TRH did not evoke oscillations in non-oscillating melanotropes nor did it have any effect on baseline $[Ca^{2+}]_i$ (Fig. 5C, N=6).

Sauvagine

Sauvagine increased the oscillation frequency of all spontaneously oscillating melanotropes by 158 ± 25 % (mean ± S.D.) when added at a concentration of 1 nM (Fig 6A, N=18). After 5 min superfusion with sauvagine an increase in baseline $[Ca^{2+}]_i$ was observed in 10 out of 18 cells. In 2 out of 6 non-oscillating cells sauvagine evoked low amplitude oscillations and induced a slight elevation of baseline $[Ca^{2+}]_i$ (Fig. 6B).

DISCUSSION

Spontaneous Ca^{2+} oscillations are under external control

In this study we confirm our recent demonstration that approximately 80% of the melanotropes isolated from black-adapted toads of the species *Xenopus laevis* display spontaneous Ca^{2+} oscillations [22]. In culture, melanotropes of white-adapted animals become activated, due to the loss of inhibitory hypothalamic control, and such cells also show spontaneous Ca^{2+} oscillations [W.J.J.M. Scheenen and F.J.C. van Strien, unpublished observation]. Therefore, only cells from black-adapted animals were considered in the present study. We show that dopamine, isoguvacine ($GABA_A$ agonist, at a concentration of 100 μM), baclofen ($GABA_B$ agonist) and NPY evoked a rapid quenching of the

spontaneous Ca^{2+} oscillations, whereas sauvagine induced oscillatory activity in 33 % of the non-oscillating cells and was able to increase the oscillation frequency in oscillating cells. We therefore conclude that the oscillations are under the control of neural factors previously shown to regulate the rate of spontaneous secretion of α-MSH [10].

Arguments in favour of involvement of oscillations in the regulation of sustained secretion

Since the sustained spontaneous release of α-MSH exhibited by melanotrope cells *in vitro* depends on the entry of Ca^{2+}_e [29], as do the spontaneous Ca^{2+} oscillations [22], it seems feasible that neural factors regulate sustained secretion from these cells by acting on these oscillations. The following 5 arguments emerging from the present study support this view.

(1) All the secreto-inhibitory factors tested are able to block spontaneous Ca^{2+} oscillations. The inhibitory action of baclofen, isoguvacine and dopamine was previously demonstrated in superfusion studies with both neurointermediate lobes and isolated melanotropes [27,29,31]. As to NPY, the present finding of its direct inhibitory effect on oscillatory activity of melanotropes is not consistent with our earlier proposal that this neuropeptide acts indirectly on melanotropes via folliculo-stellate cells [2,5]. Re-examination of previous experiments [2] revealed that the lack of a direct effect of NPY on isolated melanotropes was due to the fact that NPY was effectively bound to a cellulose ester filter placed on the input side of the superfusion chamber [12, W.J.J.M. Scheenen, unpublished results], a finding which is in accordance with a recent study by Kongsamut et al. [13]. Consequently, the inhibitory action of NPY on Ca^{2+} oscillations observed in the present study fully correlates with the action of NPY on α-MSH secretion.

(2) The tested secretagogues showed a remarkable similarity with respect to the degree of reversibility of their effects on oscillations and on secretion. Whereas the inhibitory effect of NPY on Ca^{2+} oscillations was not readily reversible, dopamine- and baclofen-induced inhibitions immediately disappeared upon washing. This difference in reversibility correlates with our previous finding that a short exposure to NPY causes a long-lasting inhibition of α-MSH secretion whereas inhibitions of secretion caused by dopamine and baclofen are rapidly reversible [2,15].

(3) The secreto-stimulatory action of sauvagine [28] correlates with its ability to evoke oscillations in non-oscillating cells.

(4) TRH was unable to induce oscillations in non-oscillating cells. This correlates with the fact that it does not stimulate α-MSH secretion from lobes taken from black-adapted animals [30].

(5) While in other cell types baseline $[Ca^{2+}]_i$ rather than the calcium oscillations is the focal point of regulation of sustained secretion [16,23], this does not seem

to be the case in *Xenopus* melanotropes because, while some of the secretagogues tested had an influence on baseline $[Ca^{2+}]_i$, this effect did not always correlate with the effects of the secretagogues on α-MSH secretion. In particular, the secreto- inhibitors dopamine and baclofen only occasionally lowered the baseline $[Ca^{2+}]_i$ whereas NPY had no effect and isoguvacine (at a high concentration) even increased baseline $[Ca^{2+}]_i$.

Taken together, these 5 arguments indicate that regulation of oscillatory activity is a main factor in the control of sustained secretion from *Xenopus* melanotrope cells (see also [24]). In addition to sustained secretion, melanotrope cells may release their products in another mode, viz. by short-duration phasic secretion. This type of secretion can be induced when isoguvacine is administered in a low (1-10 μM) concentration [31] and it has recently been shown that such phasic secretory responses to isoguvacine can not be blocked by the chloride-channel blocker picrotoxin [11]. Assuming that cytosolic Ca^{2+} dynamics play a role in the control of this type of release, three aspects of these dynamics investigated in the present study could theoretically control this release: the calcium oscillations *per se*, the frequency of the oscillations, or single large Ca^{2+} transients observed following isoguvacine treatment. The fact that picrotoxin blocks isoguvacine-induction of oscillations but not the induction of a single large Ca^{2+} transient, suggests that this transient is more directly coupled to the phenomenon of phasic release than are the oscillations. Transients not only occur in non-oscillating cells but also in oscillating cells when exposed to TRH. As TRH does not evoke a phasic release from such cells [30], the function of the transient in oscillating cells remains obscure.

Some secretagogues increased oscillation frequency of oscillating cells (sauvagine, isoguvacine at low concentrations) but there is no clear correlation between this action and sustained secretion: while sauvagine is established to increase sustained secretion [28], isoguvacine stimulates only phasic secretion [31]. The exact relationship between cytosolic Ca^{2+} dynamics and secretion in the *Xenopus* melanotrope cell awaits future studies on single melanotropes in which measurements of $[Ca^{2+}]_i$ and of hormone release will be combined.

Possible mechanisms of neural control of Ca^{2+} dynamics

The question arises as to the mechanisms by which the various neural factors exert their effects on $[Ca^{2+}]_i$. Previous studies on the regulation of the secretory responses showed that dopamine- and $GABA_B$-receptor activations involve a G_i-protein, whereas sauvagine acts on a G_s protein-coupled receptor [3]. In *Xenopus* melanotropes spontaneous Ca^{2+} oscillations completely depend on Ca^{2+} entry through (ω-conotoxin-sensitive) voltage-operated Ca^{2+} channels [22,25]. Although little is known about the mechanism underlying the spontaneous opening of these channels, opening may be the result of spontaneous

membrane depolarizations. Such depolarization can occur by activation of aspecific cation channels, as has been described for melanotropes of *Rana ridibunda* [26]. Secreto-inhibitors most likely influence oscillatory activity by inhibiting, either directly or indirectly, N-type Ca^{2+} channels. The isoguvacine-induced single large transient may come about through transitory opening of such channels. In the case of TRH-induced transients it has been established for other cell types, including the melanotrope of the amphibian *R. ridibunda*, that TRH stimulates the production of inositol trisphosphate which in turn mobilizes Ca^{2+} from intracellular stores [6]. Possibly, the Ca^{2+} transient induced by TRH in oscillating *Xenopus* melanotropes reflects such an action. We have previously shown that in *Xenopus* melanotropes thapsigargin-sensitive intracellular Ca^{2+} stores are present but are not directly involved in maintaining the spontaneous oscillations [22].

Cellular heterogeneity underlying physiological recruitment of melanotrope cells

The present study shows that, for most secretagogues, individual melanotropes respond with different sensitivities. This heterogeneity in sensitivity manifests itself in the number of cells that respond with initiation or cessation of oscillatory activity when challenged with a secretagogue. Such differences in sensitivity of endocrine cells for regulatory factors have also been reported for rat lactotropes [1,17,18] and, more recently, for *Xenopus* melanotropes [24]. This heterogeneity likely forms the basis for recruitment to, and de-recruitment from the active state as seen in melanotropes during physio- logical adaptation of *Xenopus laevis* to a changed background light intensity [4].

Conclusion

The present study supports the hypothesis that Ca^{2+} oscillations in melanotropes of *Xenopus laevis* are under inhibitory and stimulatory control by neural messengers. In their reactions, the individual cells show heterogeneity which may underlie a physiological recruitment mechanism by which the cells respond to a changed physiological demand for α-MSH. The results are furthermore in line with the idea that the occurrence of Ca^{2+} oscillations in melanotropes is a key factor in setting the level of sustained secretory activity.

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NEUROPEPTIDE Y INHIBITS Ca^{2+} OSCILLATIONS, CYCLIC AMP AND SECRETION OF MELANOTROPE CELLS OF *XENOPUS LAEVIS* VIA A Y_1 RECEPTOR

With Helger G Yntema, Peter H G M Willems, Eric W Roubos and Bruce G Jenks
Peptides, *in press*

The melanotrope cells in the pituitary gland of *Xenopus laevis* are innervated by neurons containing neuropeptide Y (NPY). In the present study the mechanism of action of NPY on the melanotropes has been studied. NPY inhibited *in vitro* secretion from melanotropes in intact neurointermediate lobes as well as from isolated, single melanotropes. Inhibition of secretion from neurointermediate lobes was mimicked by the NPY analogs PYY and (Leu^{31},Pro^{34})-NPY, whereas NPY(13-36) was inactive. Secretion from isolated melanotropes was inhibited by (Leu^{31},Pro^{34})-NPY and NPY(13-36) but NPY(13-36) was 10-fold less potent than (Leu^{31},Pro^{34})-NPY. Studies on isolated cells revealed that NPY and its analogs inhibited the occurrence of intracellular Ca^{2+} oscillations with the same potency as they inhibited secretion from isolated cells. In addition to inhibiting basal secretion and spontaneous Ca^{2+} oscillations, NPY inhibited the basal production of cyclic AMP. On the basis of these results it is proposed that NPY inhibits secretion from *Xenopus* melanotropes by inhibiting cyclic AMP-dependent spontaneous Ca^{2+} oscillations through a Y_1-like receptor.

Melanotrope cells in the pars intermedia of the pituitary gland of the amphibian *Xenopus laevis* convert neural input by classical neurotransmitters and neuropeptides into a hormonal output, viz the secretion of α-melanophore stimulating hormone (α-MSH) This hormone causes dispersion of pigment in dermal melanophores resulting in darkening of the skin [14,15] Recently, particular attention has been paid to the cellular signaling mechanisms that transduce the neural inputs into α-MSH secretion Corticotropin releasing hormone (CRH) stimulates α-MSH secretion by stimulating adenylyl cyclase [8], whereas thyrotropin releasing hormone (TRH) stimulates this secretion by enhancing the production of inositol trisphosphate [11] On the other hand, melanotropes also receive various inhibitory inputs It has been shown that γ-aminobutyric acid (GABA) inhibits α-MSH secretion via two receptor subtypes, a chloride channel containing a $GABA_A$ receptor, and a G-protein-coupled $GABA_B$ receptor that inhibits adenylyl cyclase [8,15] Dopamine inhibition of α-MSH secretion is achieved via a D_2- like receptor coupled to a G-protein and inhibiting adenylyl cyclase [8,15] The present study is concerned with the cellular signal transduction pathway by which neuropeptide Y (NPY) inhibits α-MSH secretion The inhibitory effect of NPY is very characteristic as it lasts for many hours after removal of NPY, in contrast to the short-lasting inhibitions evoked by GABA and dopamine [17] Recent studies have revealed that NPY is also a very potent blocker of spontaneously occurring intracellular Ca^{2+} oscillations in isolated *Xenopus* melanotropes kept *in vitro* [25, 27] Several studies in central and peripheral tissues indicate that NPY acts in both mammals and non-mammals [6,18,22], generally acting via inhibition of adenylyl cyclase activity [1,18,19,29,34] For *Xenopus* melanotropes it was found that spontaneous Ca^{2+} oscillations depend on Ca^{2+} influx through ω-cono-

toxin-sensitive Ca^{2+}-channels [24,28]. Furthermore, inhibition of the c-AMP-dependent protein kinase led to an inhibition of Ca^{2+} oscillations [27]. Therefore, in the present study we have investigated the possibility that NPY inhibits α-MSH secretion from *Xenopus* melanotropes by binding to a specific receptor that inhibits c-AMP production and the occurrence of the Ca^{2+} oscillations.

First, the NPY receptor subtype involved in inhibition of α-MSH secretion was identified. At least three receptor subtypes have been described for NPY, as reviewed by Michell [18]. The subtypes can be distinguished on the basis of their differential responses to agonistic NPY-analogs; the Y_1-receptor subtype is activated by (Leu^{31},Pro^{34})-NPY but not by NPY(13-36), whereas, conversely, the Y_2-receptor subtype is activated by NPY(13-36) but not by (Leu^{31},Pro^{34})-NPY [3, 18]. Peptide YY (PYY) activates both the Y_1 and the Y_2 receptor but has no effect on the Y_3-receptor [18]. In order to determine the receptor subtype that is responsible for the inhibitory action of NPY on *Xenopus* melanotropes, the effects of NPY and NPY analogs on α-MSH secretion from superfused neurointermediate lobes were studied. Furthermore, to substantiate the hypothesized relation between spontaneous Ca^{2+} oscillations and hormone secretion [25,27], the effects of NPY and the analogs on oscillations and secretion were investigated with *in vitro* superfusion and dynamic video imaging techniques, using isolated, single melanotropes. Finally, the possible involvement of c-AMP in NPY-induced inhibition of α-MSH secretion was tested by determining the effects of a membrane permeable c-AMP analog on spontaneous Ca^{2+} oscillations, and by measuring c-AMP release from neurointermediate lobes.

MATERIALS AND METHODS

Animals

Young-adult *Xenopus laevis* were taken from laboratory stock and adapted to a black background for three weeks under continuous illumination, at 22 °C They were fed weekly with beef heart

In vitro secretion studies with neurointermediate lobes

Neurointermediate lobes were rapidly dissected and placed in 10 µl superfusion chambers Four chambers were superfused simultaneously with incubation medium (IM) consisting of 112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 15 mM Ultral-HEPES (Calbiochem, La Jolla CA, USA, pH 7 4), 2 mg/ml glucose, 0 3 mg/ml bovine serum albumin (Sigma, St Louis MO, USA) and 1 µg/ml ascorbic acid, at a rate of 1 5 ml/h, at 22 °C In order to establish stable release, lobes were superfused for at least 75 min (10 fractions of 7 5 min) with IM before NPY and its analogs were added The concentration of α-MSH in each fraction was determined by radioimmunoassay [31] Basal (spontaneous) α-MSH release was defined as the average secretion in the three fractions preceding the first application. Details on concentrations of the NPY analogs used and their administration protocols are given in the Results section In experiments in which c-AMP secretion was measured, superfusion was

performed in the presence of 0 1 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma) and fractions were collected every 15 min [8]

Preparation of single melanotropes

Isolation of melanotrope cells was performed as described previously [24] In short, after anaesthetization in 0 1 % MS222 (Sigma) animals were perfused with *Xenopus* Ringer's solution, containing 112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$ and 15 mM Ultral-HEPES, to remove blood cells Then neurointermediate lobes were dissected and incubated for 45 min in Ringer's solution without $CaCl_2$ to which 0 25 % (w/v) trypsin (Gibco, Renfrewshore, UK) had been added Cells were subsequently dispersed in Leibovitz's L15 medium, which has been adjusted to *Xenopus* blood osmolality (L15 ultrapure water = 2 1) and contained 10 % fetal calf serum (Gibco) Single cells were plated on glass cover slips coated with poly-L-lysine (Sigma; Mw > 300 kD) at a density of 10,000 cells/slip At least 95 % of the cells prepared in this way are melanotropes [9]

Measurement of peptide release from single melanotropes

To measure secretion from single cells, freshly isolated cells were cultured in 75 µl IM containing 22 5 µCi ^{3}H-lysine (86 Ci/mmol, Amersham, Buckinghamshire, UK) for 18 h After washing, cover slips were placed in a superfusion chamber with a volume of 200 µl The superfusion flow rate was 1 ml/min Cells were superfused for 1 5 h before NPY analogs were added and 1 min fractions were collected To each fraction 1 ml scintillation fluid (Optiphase 'HiSafe' 3, LKB-Wallac, Turku, Finland) was added and radioactivity was determined using a ß-counter (1216 Rackbeta, LKB-Wallac)

Radioimmunoassays

Radioimmunoassay for α-MSH was performed as described previously, using an antiserum raised in our laboratory [31], which has equal affinity for the acetylated and non-acetylated forms of α-MSH Cross-reactivities with ACTH (1-24) and ACTH (1-39) were below 0 01% Bound and free antibodies were separated by polyethylene glycol/ovalbumin precipitation. Detection limit was 2 pg α-MSH per sample Superfusion fractions were assayed in duplicate Radioimmunoassay for c-AMP was performed as described previously [8], using a c-AMP-RIA kit from Amersham Detection limit was 1 fmol c-AMP per sample.

Ca^{2+} measurements

Ca^{2+} measurements in isolated melanotrope cells were performed as described previously [24,25] In short, single cells were cultured for three days at 22 °C. After culturing, cells were loaded with 2 µM Fura-2/AM (Molecular Probes, Eugene OR, USA) in IM containing 1 µM pluronic F127 [23] (Molecular Probes) for 20 min at 22 °C After loading, cells were washed with IM in a Leiden perifusion chamber [13] (volume 800 µl) at a rate of 1 ml/min, for 25 min During this wash unattached cells were sucked off and attached cells were allowed to equilibrate. The chamber was placed on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan) and the light from a 100 W xenon lamp was directed through a quartz neutral density filter (ND 2, Ealing Electro-Optics, Holliston, MA, USA) to reduce bleaching of the intracellularly trapped fluorochrome The excitation bandpass filters, mounted in a motordriven rotating wheel, had transmission maxima at 340 and 380 nm (± 12 nm) (Ealing Electro-Optics) The fluorescence emission ratio at 492 nm was used as a measure of $[Ca^{2+}]_i$ after excitation at 340 and 380 nm [21]. An epifluorescent 40x magnification oil immersion

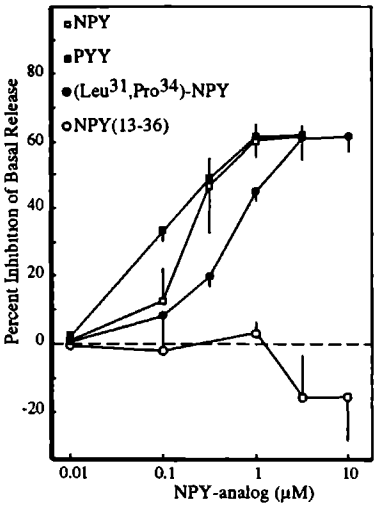

Figure 1: Dose-response relationship of NPY analogs on α-MSH secretion from neurointermediate lobes. The efficacies of PYY and NPY were similar; both analogs produced a maximal inhibition of 60 % at 1 μM. (Leu[31], Pro[34])-NPY was less potent that NPY and PYY at 0.1 and 1 μM, but eventually reached an similar efficacy at 3.3 μM. NPY(13-36) did not inhibit α-MSH secretion at any concentration tested. At 3.3 and 10 μM NPY(13-36) caused a stimulation of 16 %.

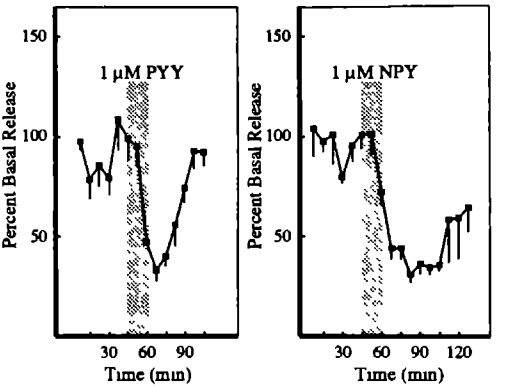

Figure 2: Reversibility of PYY- and NPY-induced inhibition of α-MSH secretion in freshly dissected neurointermediate lobes. The PYY-induced inhibition was completely reversed within 5 superfusion fractions (37.5 min) after removal of the test substance. NPY-induced inhibition was only reversed by 45 % after 9 superfusion fractions (67.5 min).

objective was used Dynamic video imaging was carried out using the MagiCal hardware and TARDIS software of Joyce Loebl (Dukesway, Tyne & Wear, UK) as described by Neylon et al [20]. The inter- frame interval between the ratio frames was 6.4 s with a maximal sampling time of 32 min Test substances were added to the bath using a perifusion pump.

Pharmaca

Substances tested were NPY, NPY(13-36), PYY (Bachem Feinchemikalien, Bubendorf, Switzerland) and (Leu[31],Pro[34])-NPY (Bachem California, Torrance CA, USA). 8-Br-c-AMP was obtained from Sigma. In some experiments a test-pulse of dopamine (Sigma) was included.

Calculations and statistics

Results of superfusion experiments with neurointermediate lobes are shown as the average ± SEM of four lobes superfused in one experiment, unless stated otherwise Each experiment was performed at least 2 times independently. Percentages of inhibition and stimulation of α-MSH- or c-AMP-release were calculated on the basis of integration of the respective peak areas in the graphs, using three fractions preceding and three fractions following the onset of experimental treatment. In superfusion experiments with isolated melanotropes pulses of 5 min were given (5 fractions), and the calculation was made using the

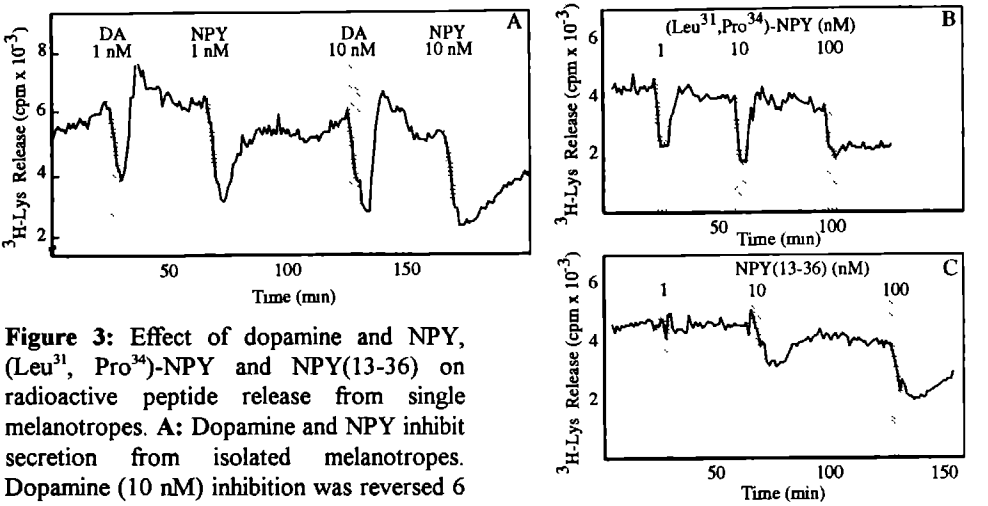

Figure 3: Effect of dopamine and NPY, (Leu^{31}, Pro^{34})-NPY and NPY(13-36) on radioactive peptide release from single melanotropes. **A:** Dopamine and NPY inhibit secretion from isolated melanotropes. Dopamine (10 nM) inhibition was reversed 6 min after dopamine removal; removal of 10 nM NPY caused a very slow reversal of the inhibition which was not completed after 30 min. **B:** Dose-response relationship of (Leu^{31}, Pro^{34})-NPY induced inhibition. 1 nM caused an inhibition of 45 %; maximum inhibition (55 %) was reached at 10 nM; application of 100 nM did not increase the magnitude of the inhibition but caused a long-lasting inhibition. **C:** Dose-response relationship of NPY (13-36). 1 nM did not inhibit secretion; 10 nM resulted in an inhibition of 35 % and 100 nM caused a long-lasting inhibition of 50 %.

five fractions before and the five fractions after the onset of treatment. The integrated areas were analyzed with the paired Student's T- test. A p-value <0.05 was considered to indicate statistical significance.

RESULTS

Effects of NPY and NPY-analogs on α-MSH secretion from neurointermediate lobes

NPY dose-dependently inhibited basal α-MSH secretion with a minimum effective concentration of 0.1 μM, leading to an inhibition of 12 ± 8 %. Maximum inhibition (60 ± 7 %) was achieved at a concentration of 1 μM (Fig. 1). PYY was slightly more potent than NPY in inhibiting α-MSH secretion, giving 33 ± 3 % inhibition at a concentration of 0.1 μM (Fig. 1). Although the efficacy, achieved over a period of 22.5 min, was similar for NPY and PYY, the

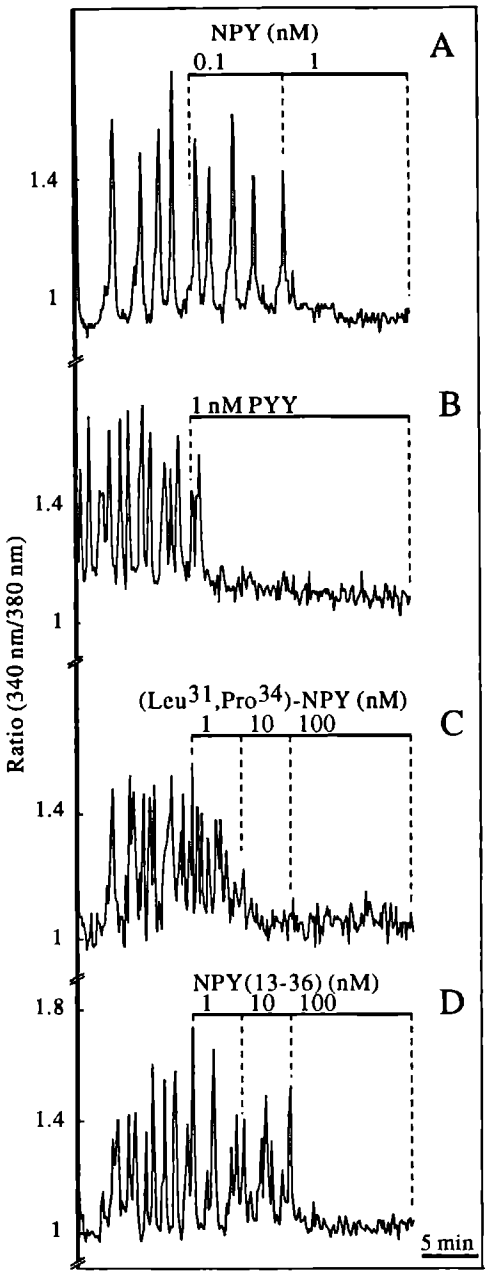

Figure 4: Effects of NPY, PYY and (Leu^{31}, Pro^{34})-NPY on spontaneous Ca^{2+} oscillations. **A:** NPY inhibits spontaneous oscillations in all cells studied at a concentration of 1 nM. **B:** PYY inhibits the oscillations in all cells at 1 nM. **C:** (Leu^{31},Pro^{34})-NPY inhibits the oscillations in 22 % of the cells at 1 nM. To achieve an inhibition in all cells a concentration of 10 nM had to be applied. **D:** NPY(13-36) inhibits oscillations in all cells at a concentration of 100 nM. At 10 nM oscillations were blocked in 5 out of 13 cells.

recovery from inhibition clearly differed; NPY had a long-lasting effect (more than one hour) whereas PYY-induced inhibition fully disappeared within 37.5 min following PYY removal (5 superfusion fractions) (Fig. 2). (Leu^{31},Pro^{34})-NPY inhibited α-MSH secretion at a minimal effective concentration of 0.1 μM, giving 10 ± 6 % inhibition, and maximum inhibition (60 ± 7%) was achieved at a concentration of 3.3 μM (Fig. 1). NPY (13-36) was ineffective in inhibiting α-MSH secretion in concentrations up to 10 μM (Fig. 1).

Effects of NPY and NPY-analogs on secretion from single cells

During a 5 min pulse NPY inhibited release of radioactive peptides from single cells cultured on coated cover slips by 50 % when applied at a concentration of 1 nM (Fig. 3A). At 10 nM the percentage of inhibition was 60 %. Compared to the dopamine-induced inhibition, recovery from inhibition with NPY was long lasting after removal of the inhibitor. Both NPY-analogs inhibited secretion; after removal of the inhibitor the inhibition by (Leu^{31},Pro^{34})-NPY was

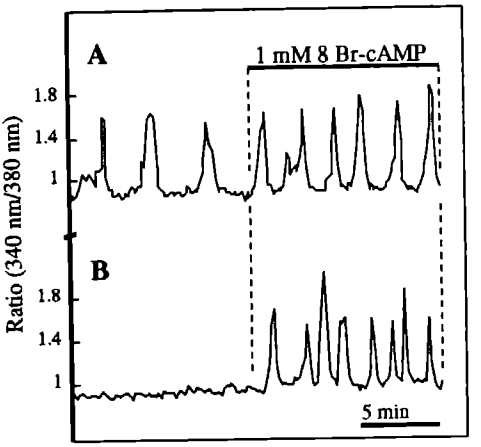

Figure 5: Effects of 8-Br-c-AMP on Ca^{2+} oscillations. **A:** 8-Br-c-AMP (1 mM) strongly increased the frequency of spontaneous oscillations (120 %). **B:** In 6 out of 8 non-oscillating cells 1 mM 8-Br-c-AMP induced oscillations.

readily reversible whereas the effect of NPY(13-36) was long lasting. At 1 nM (Leu^{31}, Pro^{34})-NPY inhibited secretion by 45 % (Fig. 3B); when a concentration of 10 nM was given maximum inhibition was reached (55 %) (Fig. 3B). 1 nM of NPY(13-36) had no effect on secretion; 10 nM inhibited secretion by 35 % and 100 nM NPY(13-36) induced an inhibition of 50 % (Fig. 3C).

Effects of NPY and NPY-analogs on spontaneous Ca^{2+} oscillations in single melanotropes

Both NPY and PYY appeared to be very potent in blocking spontaneous Ca^{2+} oscillations in single melanotropes. Complete inhibition by NPY was reached at a concentration of 1 nM (Fig. 4A, N=29). PYY was similarly effective in inhibiting the spontaneous Ca^{2+} oscillations, causing a complete inhibition at 1 nM (Fig. 4B, N= 11). (Leu^{31},Pro^{34})-NPY completely inhibited spontaneous Ca^{2+} oscillations at a concentration of 10 nM (Fig. 4C, N= 18) whereas in 4 cells oscillations were already blocked at 1 nM. NPY (13-36) inhibited oscillations in 5 out of 13 cells when tested at a concentration of 10 nM. Complete inhibition of the oscillations in all cells was achieved by 100 nM NPY(13-36) (Fig. 4D, N= 11).

Effect of 8-Br-c-AMP on Ca^{2+}- oscillations in single melanotropes

The cell-permeable c-AMP-analog 8-Br-c-AMP increased the frequency of spontaneous Ca^{2+} oscillations by 134 ± 12 % when applied at 1 mM (Fig. 5A, N= 33). 1 mM 8-Br-c-AMP induced oscillations in 6 out of 8 non-oscillating cells (Fig. 5B).

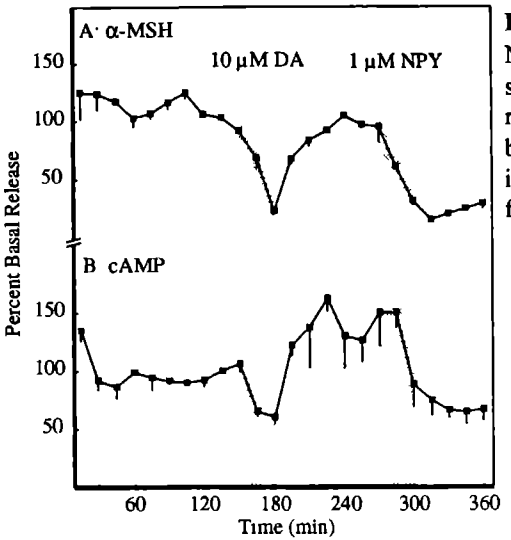

Figure 6: Comparison of dopamine- and NPY-induced inhibition of the α-MSH secretion (**A**) and c-AMP release (**B**) in neurointermediate lobes A strong correlation between inhibition of α-MSH secretion and inhibition of c-AMP efflux was noted for both factors.

Effect of NPY on c-AMP release from neurointermediate lobes

When 1 μM NPY was added to the superfusion medium in the presence of the phosphodiesterase inhibitor IBMX, α-MSH secretion was reduced by 60 ± 5 %. Parallel to this inhibition, an inhibition of c-AMP secretion of 55 ± 13 % was found (Fig. 6). In the same experiment, a pulse of dopamine was given as this is known to exert a well characterized effect on c-AMP production [8]. Dopamine produced 55 ± 8 % inhibition of α-MSH secretion and 40 ± 6 % inhibition of c-AMP secretion. The dopamine-induced inhibition had fully disappeared within 30 min following dopamine removal (2 superfusion fractions) whereas the inhibition of NPY was long-lasting: even after one hour (4 superfusion fractions) the release of α-MSH and c-AMP had not returned to basal levels.

DISCUSSION

In the present study we investigated the action mechanism by which NPY inhibits secretory activity of *Xenopus* melanotropes. The finding that NPY is a potent blocker of secretion from melanotropes in the neurointermediate lobe is consistent with earlier studies [32]. From the studies on the action of NPY analogs on α-MSH secretion from neurointermediate lobes, showing that NPY, PYY and (Leu^{31},Pro^{34})-NPY inhibit secretion whereas NPY(13-36) does not, we conclude that a Y_1-like receptor subtype is involved. The finding that PYY is slightly more potent than NPY and (Leu^{31},Pro^{34})-NPY is slightly less potent than

NPY is in agreement with the different potencies of these analogs to agonize Y_1-receptors reported in other studies [3,18].

NPY acts with a long duration on *Xenopus* melanotropes as is clear from the fact that the recovery from inhibition by neurointermediate lobes and single melanotropes is slow. This action seems to be specific for NPY as it was not displayed by PYY. The most likely explanation for this phenomenon is that the binding of NPY to its receptor is less reversible than that of PYY.

The pharmacological characteristics of the NPY receptor responses found in our secretory studies with cultured melanotropes differed from the strict Y_1 classification found for freshly dissected neurointermediate lobes. While the Y_1-receptor agonist (Leu^{31}, Pro^{34})-NPY was effective both in fresh tissue and cultured single melanotropes, the cultured cells also showed an inhibitory secretory response to NPY(13-36), a NPY analog that specifically activates a Y_2-receptor subtype. In other tissues, a 100- to 1000-fold difference in potency between these Y_1- and Y_2-receptor agonists for the Y_1-receptor has been reported [3,18]. The fact that, in our experiments with cultured *Xenopus* melanotropes, only a 10-fold difference was found might indicate that with culturing there is induction of a Y_2-receptor. Alternatively, the results could reflect changes in Y_1 receptor structure as a result of cell culture and/or trypsinization. Changes in receptor structure after trypsinization and subsequent changes in receptor affinity for ligands, has been reported for IgG receptors in monocytes [30]. Since the Y_1 receptor agonist is more potent than the Y_2 receptor agonist and since for fresh lobes no Y_2 effect was found, we conclude that a Y_1-like receptor is primarily involved in the inhibition of α-MSH secretion *in vivo*. We further suggest that the Y_2-receptor agonist activity, seen in the present study and also reported by Kongsamut et al. [16], reflects an artifact.

It was apparent that the secretory activity of cells cultured on poly-l-lysine-coated cover slips was a 100 times more sensitive to the inhibitory action of NPY and its analogs than that of the freshly dissected tissue. Possible explanations for this phenomenon are (1) an increase in density of receptor on cultured cells and (2) a relatively poor accessibility of the melanotropes in intact lobes for exogenously applied NPY. This accessibility could be hampered by, for instance a relatively poor penetration of NPY into the tissue, the uptake of NPY by glial cells or extracellular enzymatic degradation of NPY. It should be noted that *in vivo* NPY reaches the melanotrope via specialized local synaptic contacts so that the peptide may locally reach relatively high concentrations [10].

Testing NPY and its analogs on intracellular Ca^{2+} dynamics showed that NPY as well as analogs are capable of completely inhibiting spontaneous Ca^{2+} oscillations. In secretion studies with isolated melanotropes NPY induced only 60 to 70 % inhibition of basal secretion of radiolabeled products, which seems to be contradictory to the complete inhibition of Ca^{2+} oscillations by NPY in such

cells. However, in recent studies we found that about one-third of radiolabeled products released by the single melanotropes consists of amino acids not incorporated into peptides (B.G. Jenks, unpublished observation). Therefore, we propose that NPY is able to inhibit completely both peptide secretion and Ca^{2+} oscillations.

In Ca^{2+} measurements, the Y_1-agonist was more potent than the Y_2-agonist in inhibiting the occurrence of spontaneous Ca^{2+} oscillations. The observation that the potency of the NPY-analogs was equal for inhibiting single cell secretion and Ca^{2+} oscillations supports earlier arguments that spontaneous Ca^{2+} oscillations are the driving force for *in vitro* α-MSH secretion [25,27].

NPY receptors have been demonstrated to inhibit the adenylyl cyclase system in various cell types [1,18,19,29,34] but there are also reports that Y_1-receptor activation can lead to an increase in intracellular [Ca^{2+}] [1,7,29,33] and that the Y_2-receptor directly inhibits a (N-type) voltage-operated Ca^{2+} channel [2,4,5,12,33]. To assess whether NPY receptor activation in *Xenopus* melanotropes leads to a decreased c-AMP production, we investigated the effect of NPY on c-AMP efflux. This efflux can be taken as a measure for the intracellular c-AMP concentration [8]. The results show that NPY decreases c-AMP efflux from *Xenopus* melanotropes, indicating that NPY inhibits the production of c-AMP. This finding is in agreement with the action of NPY in most cell systems studied.

For *Xenopus* melanotropes it has been reported that inhibition of c-AMP-dependent protein kinase leads to an abolishment of spontaneous Ca^{2+} oscillations [26]. Our data, showing that a cell-permeable c-AMP-analog can increase the frequency of spontaneously occurring Ca^{2+} oscillations and can induce oscillations in non-oscillating cells [see also 26] support the idea that in *Xenopus* melanotropes a c-AMP-dependent phosphorylation is involved in the induction of spontaneous Ca^{2+} oscillations. On the basis of the present findings we suggest that in *Xenopus* melanotropes NPY inhibits c-AMP production via Y_1 receptors, leading to inhibition of spontaneous Ca^{2+} oscillations and subsequently to inhibition of α-MSH secretion.

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DIFFERENTIAL REGULATION OF INTRACELLULAR CALCIUM OSCILLATIONS BY SECRETO-INHIBITORS IN MELANOTROPE CELLS OF *XENOPUS LAEVIS*

With Peter H G M Willems, Renier J A M van Dinter, Bruce G. Jenks and Eric W Roubos

submitted

The secretion of α-melanotrope stimulating hormone (α-MSH) from melanotrope cells of *Xenopus laevis* is regulated by multiple inhibitory factors, namely dopamine, neuropeptide Y and γ-aminobutiric acid (GABA), which inhibits through both $GABA_A$ and $GABA_B$ receptors. Spontaneous Ca^{2+} oscillations observed *in vitro* in melanotropes probably drives secretion. The present study focuses on possible differential mechanisms by which secreto-inhibitors inhibit Ca^{2+} oscillations. To assess the involvement of a hyperpolarization mechanism in receptor-mediated control of Ca^{2+} oscillations the effects of depolarizing pulses of high $[K^+]$ were tested on Ca^{2+} oscillations and baseline $[Ca^{2+}]$ in single melanotropes that were inhibited by the secreto-inhibitors. The contribution of L-type and N-type voltage-activated Ca^{2+} channels to the K^+-response was assessed by using nifedipine and ω-conotoxin GVIA. Inhibition of Ca^{2+} oscillations through the $GABA_A$ receptor agonist isoguvacine could not be overcome by 20 mM K^+. Upon stronger depolarization (60 mM K^+) some oscillatory activity returned, indicating a strong hyperpolarizing action of the $GABA_A$ receptor. In melanotropes inhibited with the $GABA_B$ receptor agonist baclofen 20 mM K^+ induced Ca^{2+} oscillations in all cells, whereas the K^+-channel blocker tetraethyl ammonium (TEA) had no effect on baclofen-induced inhibition of Ca^{2+} oscillations. These results indicate no role for a TEA-sensitive K^+ channel in $GABA_B$ receptor-induced inhibition of the cell and we propose that the main mechanism by which the $GABA_B$ receptor inhibits Ca^{2+} oscillations is by a c-AMP-dependent pathway. In dopamine-inhibited cells 20 mM K^+ only induced Ca^{2+} oscillations when these cells had been pretreated with TEA, indicating that the D_2-receptor mechanism inhibits not only through inhibitory action on adenylyl cyclase but also through membrane hyperpolarization by activation of TEA-sensitive K^+ channels. The action of the NPY receptor could be distinguished from both the $GABA_B$ and D_2 receptor in that only about 50 % of the NPY-inhibited cells displayed 20 mM K^+ induced oscillations. In contrast to dopamine-inhibited cells, NPY-induced inhibition could not be overcome by TEA treatment.

We conclude that the secreto-inhibitors differentially affect the ability of ω-conotoxin-sensitive N-type Ca^{2+} channels to produce Ca^{2+} oscillations. Moreover, the main site of action of the secreto-inhibitors is on the N-type channel and not the L-type channel, since K^+-induced depolarizations led to a nifedipine-sensitive elevation of the baseline Ca^{2+} concentrations in all receptor-inhibited conditions.

In recent years increasing interest is being paid to the possible significance of calcium oscillations in secretory active cells [1,2]. For melanotrope cells of the pituitary pars intermedia of the South African clawed toad *Xenopus laevis* there is circumstantial evidence that spontaneous Ca^{2+} oscillations play an important role in the induction of secretion of α-melanophore-stimulating hormone (α-MSH) [3,4]. *Xenopus* melanotropes possess at least two types of high voltage-activated (HVA) Ca^{2+} channels, namely an inactivating channel sensitive to ω-conotoxin and a non-inactivating channel sensitive to nifedipine [5]. Oscillations and secretory activity are both inhibited by blocking the ω-conotoxin-sensitive N-type channels [5,6]. This supports the idea that secretion of α-MSH is induced by Ca^{2+} oscillations that depend on influx of extracellular Ca^{2+} through N-type Ca^{2+} channels. Oscillatory and secretory activities are inhibited

by three neuroregulators, dopamine, neuropeptide Y (NPY) and γ-aminobutyric acid (GABA) [3,4,7,8]. In the present study we focus on possible differential mechanisms used by the secreto-inhibitors and their associated receptors to inhibit Ca^{2+} oscillatory activity.

In vitro superfusion experiments with *Xenopus* melanotropes have shown that dopamine and GABA cause a fast, short-lasting inhibition of α-MSH release, whereas NPY inhibits slowly but with a long-lasting effect [9]. On the basis of these data the question arises whether these regulators also act differentially on Ca^{2+} oscillations. The receptors through which these secreto-inhibitors influence the melanotropes have been identified; dopamine acts via a D_2-like receptor [10], NPY via a Y_1 receptor [11] whereas GABA activates both a $GABA_A$ and a $GABA_B$ receptor [12]. However, the processes by which these receptors influence Ca^{2+} oscillatory behavior are less clear. Probably, at least the following two processes are involved: (1) Oscillations may be inhibited by dephosphorylation of Ca^{2+} channels, as is suggested by the fact that activation of D_2, Y_1 and $GABA_B$ receptors decrease c-AMP production [11, 13] and (2), oscillations may be blocked by hyperpolarization of the plasma membrane, as $GABA_A$ receptor activation opens a picrotoxin-sensitive chloride channel [4,14], presumably leading to membrane hyperpolarization. The present study focuses on possible differential actions of the secreto-inhibitors on Ca^{2+} oscillations by assessing the significance of a hyperpolarization mechanism for receptor control of Ca^{2+} oscillations. To this end, the effects of depolarizing pulses of high $[K^+]$ were tested on single melanotropes that were inhibited by the various secreto-inhibitors. The contribution of L-type and N-type voltage-operated Ca^{2+} channels to the Ca^{2+} response was assessed using the channel blockers nifedipine and ω-conotoxin, respectively. Intracellular Ca^{2+} dynamics were visualized using video imaging microscopy and the fluorescent Ca^{2+}-indicator Fura-2. The results demonstrate that secreto-inhibitors differentially affect the sensitivity of N-type Ca^{2+} channels to K^+-evoked membrane depolarizations.

MATERIALS AND METHODS

Animals

Young-adult *Xenopus laevis* were taken from laboratory stock and kept on a black background for three weeks under continuous illumination, at 22 °C. The animals were fed weekly with beef heart.

Preparation of single cells

Isolation of melanotrope cells was performed as described previously [6]. In short, after anaesthetization with MS222 (1 g/l, Sigma, St. Louis, MO, USA) animals were perfused with *Xenopus* Ringer's solution, containing 112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$ and 15 mM Ultral-HEPES (Calbiochem, La Jolla CA, USA; pH 7.4), to remove blood cells. Then

neurointermediate pituitary lobes were dissected and incubated for 45 min in Ringer's solution without $CaCl_2$ to which 0.25 % (w/v) trypsin (Gibco, Renfrewshore, UK) had been added. Cells were subsequently dispersed in Leibovitz's L15 medium, containing 10 % fetal calf serum (Gibco), by gentle trituration of the lobes with a siliconized Pasteur's pipette. The medium had been adjusted to *Xenopus* blood osmolality (L15 : ultrapure water = 2:1). After washing, the cells were plated on glass cover slips coated with poly-L-lysine (Mw > 300 kD; Sigma) at a density of about 10,000 cells/slip, and cultured for 3 days at 22 °C. The cells were readily identified on the basis of their characteristic round shape.

$[Ca^{2+}]_i$ measurements

Measurements of intracellular Ca^{2+} ($[Ca^{2+}]_i$) were performed as described previously [6]. Cells were loaded with 2 mM Fura-2/AM (Molecular Probes, Eugene, OR, USA) in Ringer's solution containing 1 μM pluronic F127 [15] (Molecular Probes), for 20 min. at 22 °C, and then washed with Ringer's solution in a Leiden perifusion chamber [16] (volume 800 ml) at a flow rate of 1 ml/min, for 25 min., to remove non-hydrolysed dye During washing unattached cells were removed and attached cells were allowed to equilibrate. The chamber was placed on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan). The light from a 100 W xenon lamp was directed through a quartz neutral density filter (ND 2, Ealing Electro-Optics, Holliston, MA, USA) to reduce bleaching of the intracellularly trapped dye. The excitation bandpass filters (Ealing Electro-Optics), mounted in a motor-driven rotating wheel, had transmission maxima at 340 and 380 nm (± 12 nm) The fluorescence emission ratio at 492 nm was used as a measure of $[Ca^{2+}]_i$ after excitation at 340 and 380 nm [17] An epifluorescent 40x magnification oil immersion objective was used Dynamic video-imaging microscopy was carried out using the MagiCal hardware and TARDIS software of Joyce Loebl (Dukesway, UK) as described in detail by Neylon et al. [18]. The interframe interval between the ratio frames was 6 4 s with a maximal sampling time of 32 min Chemical substances and different ion concentrations were added via the perifusion pump

Chemicals

Nifedipine, dopamine and tetraethyl ammoniumchloride (TEA) were purchased from Sigma, baclofen and isoguvacine from RBI (Natick, MA, USA) and ω-conotoxin GVIA and NPY from Bachem (Bubendorf, Switzerland). In solutions with a high K^+ concentration, the NaCl concentration was reduced to maintain osmolality.

RESULTS

Spontaneous Ca^{2+} oscillations were observed in 76 % of the melanotropes. These oscillating cells formed the object of the present study. The oscillations arose from a basal level of the cytosolic Ca^{2+} concentration (baseline $[Ca^{2+}]_i$). The frequency of these oscillations was highly variable among individual melanotropes, ranging from 0.2 to 4.0 spikes per min. (see e.g. Fig. 1), but was remarkably constant for any given cell.

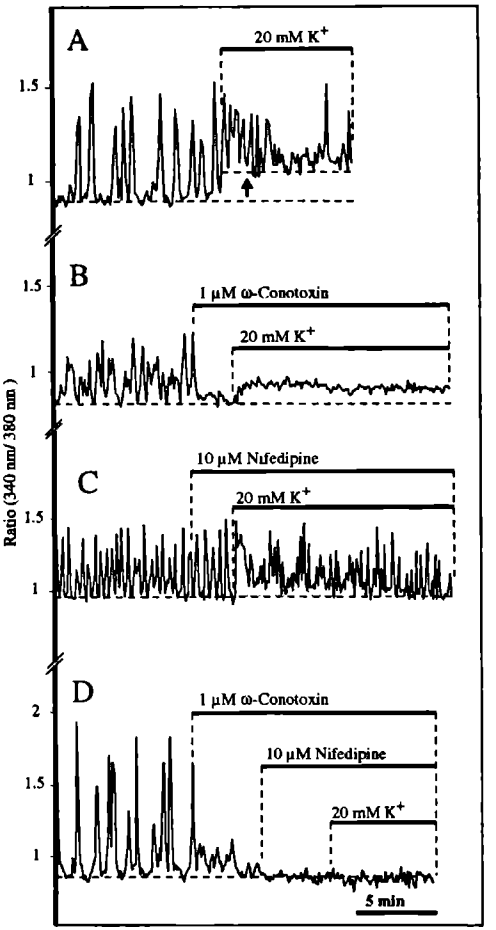


Figure 1: The effect 20 mM K^+ on Ca^{2+} dynamics in fura-2 loaded melanotropes. Fluorescence emission ratio at 492 nm is shown as a measure for $[Ca^{2+}]_i$ after excitation at 340 and 380 nm. **A**, Increasing $[K^+]_e$ from 2 to 20 mM led to a sustained increase in baseline $[Ca^{2+}]_i$ on top of which oscillations were observed. The frequency of the oscillations was increased during the first 3 min. of K^+-treatment (arrow). **B**, ω-Conotoxin (1 µM) blocked all oscillations and subsequent addition of 20 mM K^+ caused a sustained increase of baseline $[Ca^{2+}]_i$. **C**, Nifedipine (10 µM) had no effect on spontaneous Ca^{2+} oscillations, nor on baseline values and subsequent addition of 20 mM K^+ led to a slight, transient increase in baseline $[Ca^{2+}]_i$ lasting for 2 to 3 min. (shaded area) during which time oscillation frequency was increased. **D**, Addition of 20 mM K^+ to cells pretreated with a combination of 1 µM ω-conotoxin and 10 µM nifedipine had no effect on $[Ca^{2+}]_i$.

K^+-induced changes in $[Ca^{2+}]_i$

Increasing $[K^+]_e$ from 2 mM (controls; normal Ringers' solution) to 20 mM led to a sustained increase in baseline $[Ca^{2+}]_i$ and a temporary increase in oscillation frequency of 70 ± 20 % (mean ± SD.). This temporary increase in oscillation frequency lasted for about 3 min after which the frequency returned to control values (Fig. 1A, N=16). Addition of 1 µM ω-conotoxin inhibited Ca^{2+} oscillations almost immediately (Fig. 1B, N=24). In such preparations 20 mM $[K^+]_e$ induced a sustained increase in baseline $[Ca^{2+}]_i$ but oscillations did not reappear upon increasing the medium K^+ concentration. Pretreatment with 10 µM nifedipine did not prevent the occurrence of spontaneous oscillations (Fig. 1C, N=22). However, the K^+-induced elevation of baseline $[Ca^{2+}]_i$ was clearly lower in amplitude and transient compared to cells not treated with nifedipine. A combination of 1 µM ω-conotoxin and 10 µM nifedipine completely blocked the 20 mM K^+-induced elevation in baseline $[Ca^{2+}]_i$ (Fig. 1D, N=16).

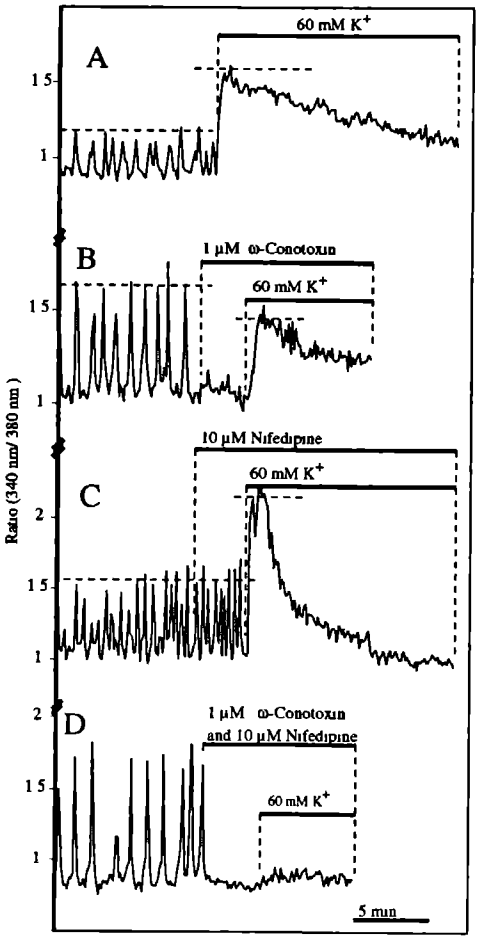
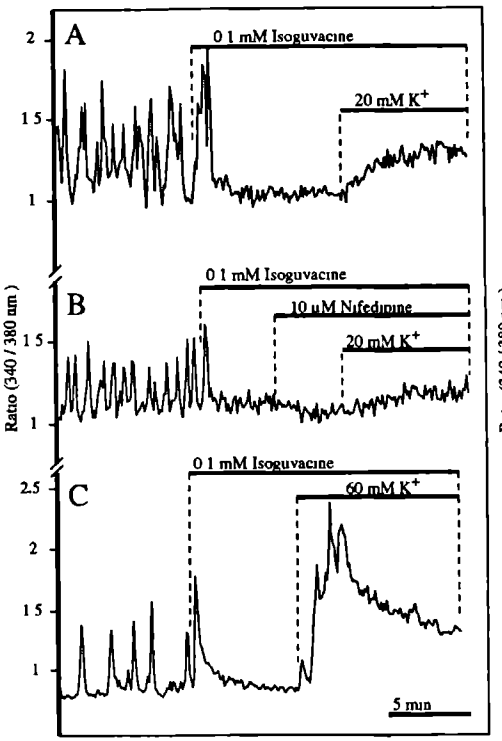


Figure 2: Effect of 60 mM K^+ on Ca^{2+}-influx through L- and N-type Ca^{2+} channels **A**, Increasing K^+_e to 60 mM led to a rapid increase in $[Ca^{2+}]_i$ (the rise phase), the amplitude of which was markedly increased as compared to the amplitude of the spontaneous oscillations After this increase $[Ca^{2+}]_i$ slowly decreased (the decline phase), leading to a sustained increase in baseline $[Ca^{2+}]_i$. **B**, ω-Conotoxin led to a reduction of the relative amplitude of the sustained increase in baseline $[Ca^{2+}]_i$ of the 60 mM K^+-induced transient **C**, Nifedipine caused a rapid decline phase of the 60 mM K^+-induced transient, reaching prestimulatory levels after 10 min **D**, Simultaneous application of 1 µM ω-conotoxin and 10 µM nifedipine almost completely abolished the 60 mM K^+-induced changes in $[Ca^{2+}]_i$

When $[K^+]_e$ was increased to 60 mM, a more pronounced increase in $[Ca^{2+}]_i$ (Ca^{2+} transient) was observed. This transient consisted of an initial rapid rise phase followed by a long-lasting gradual decline phase, resulting in a sustained increased $[Ca^{2+}]_i$. During this transient no oscillations occurred (Fig. 2A, N= 24). Adding 1 µM ω-conotoxin previous to 60 mM $[K^+]_e$ treatment did not prevent the occurrence of the K^+-evoked transient but decreased its amplitude which is clear when the amplitude of the transient and that of the oscillations are compared (Fig. 2B, N=5). Nifedipine (10 µM) did not affect the relative amplitude of the K^+-induced Ca^{2+}-transient. However, the decline phase was considerably faster than in control cells (Fig. 2C, N=23). The 60 mM K^+-induced Ca^{2+}-transient was almost completely abolished by simultaneous pretreatment with 1 µM ω-conotoxin and 10 µM nifedipine. However, such cells still showed a slight but consistent increase in baseline $[Ca^{2+}]_i$ upon stimulation with 60 mM K^+ (Fig. 2D, N=29).



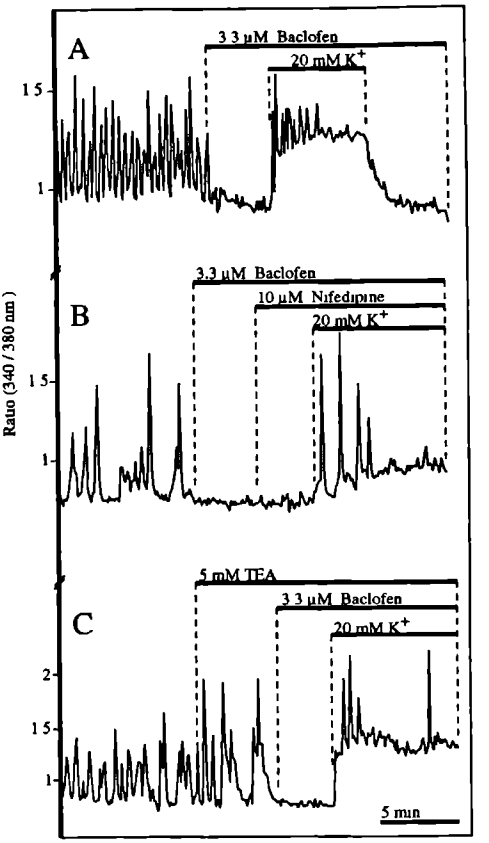


Figure 3: Effect of K^+-treatment on melanotropes inhibited by the $GABA_A$ receptor agonist isoguvacine **A**, 20 mM K^+ induced a moderate, sustained increase in baseline $[Ca^{2+}]_i$ in cells inhibited with 0 1 mM isoguvacine **B**, Addition of 10 µM nifedipine to isoguvacine-treated cells almost completely abolished the 20 mM K^+-induced increase in baseline $[Ca^{2+}]_i$ **C**, Adding 60 mM K^+ to isoguvacine-inhibited cells resulted in a rapid, strong increase in $[Ca^{2+}]_i$ During the first 3 min of K^+-treatment 2 to 3 Ca^{2+} oscillations were observed.

Figure 4: Effect of 20 mM K^+-treatment on cells treated with the $GABA_B$ receptor agonist baclofen **A**, In baclofen-inhibited cells 20 mM K^+ led to a rapid, strong increase in $[Ca^{2+}]_i$ on top of which high-frequency oscillations were observed during the first 3 min. **B**, Applying 10 µM nifedipine to baclofen-inhibited cells resulted into a gradual increase in baseline $[Ca^{2+}]_i$ upon subsequent addition of 20 mM K^+. These cells show oscillation activity during the first 3 min of K^+-treatment **C**, Treatment of cells with 0 1 mM TEA had no effect on the 3 3 µM baclofen-induced inhibition, nor on the 20 mM K^+-induced Ca^{2+} dynamics under baclofen inhibition.

Effects of secreto-inhibitors on K^+-induced changes of $[Ca^{2+}]_i$

Isoguvacine

After adding 0.1 mM isoguvacine, a single, rather large Ca^{2+} spike occurred after which the spontaneous Ca^{2+} oscillations completely stopped. A subsequent elevation of $[K^+]_e$ to 20 mM led to a gradual increase in $[Ca^{2+}]_i$ without reappearance of Ca^{2+} oscillations (Fig. 3A, N=38). This gradual increase in cytosolic Ca^{2+} level was eliminated by nifedipine (10 μM, Fig. 3B, N=20). Addition of 60 mM $[K^+]_e$ to isoguvacine-treated melanotropes resulted in a rapid increase in $[Ca^{2+}]_i$ on top of which 2 to 3 oscillations were observed (Fig. 3C, N=16).

Baclofen

Within 30 sec after application of baclofen (3.3 μM) oscillations ceased in all cells. Subsequent addition of 20 mM K^+ led to a marked increase in baseline $[Ca^{2+}]_i$ on top of which oscillations were observed for 3 min (Fig. 4A, N=38). Pretreatment with baclofen and, subsequently, nifedipine resulted in a more gradual increase in $[Ca^{2+}]_i$ in response to 20 mM K^+. Again, Ca^{2+} oscillations were observed during the first 3 min of K^+-treatment (Fig. 4B, N=28). Pretreatment with TEA (5 mM) had no effect on the 20 mM K^+-induced elevation in baseline $[Ca^{2+}]_i$ in melanotropes inhibited by baclofen nor did it affect the induction of oscillations by K^+ under these conditions (Fig. 4C, N=24).

Dopamine

Dopamine (10 nM) inhibited oscillations in all cells. When these dopamine-inhibited cells were subsequently treated with 20 mM K^+, the baseline $[Ca^{2+}]_i$ increased but the oscillations did not reappear (Fig. 5A, N=34). In dopamine-treated cells that had been subsequently exposed to 10 μM nifedipine, 20 mM K^+ did not induce an elevation in baseline $[Ca^{2+}]$ (Fig. 5B, N=17). Increasing the $[K^+]_e$ to 60 mM under dopamine treatment led to a similar Ca^{2+}-transient as observed in control cells (see Fig. 2A). Addition of 10 μM nifedipine and 10 nM dopamine before a 60 mM K^+-induced depolarization caused a transient with a fast decline phase. Moreover, Ca^{2+} spikes were superimposed on the Ca^{2+}-transient during the first 3 min (Fig. 5C, N=42).

Adding dopamine to TEA pretreated cells abolished Ca^{2+} oscillations. When such inhibited cells were given 20 mM K^+, a transient increase in baseline $[Ca^{2+}]_i$ was observed (similar to Fig. 5A) on top of which one or two Ca^{2+} spikes were observed (Fig. 5D, N=50). The occurrence of such spikes could be blocked by ω-conotoxin (data not shown).

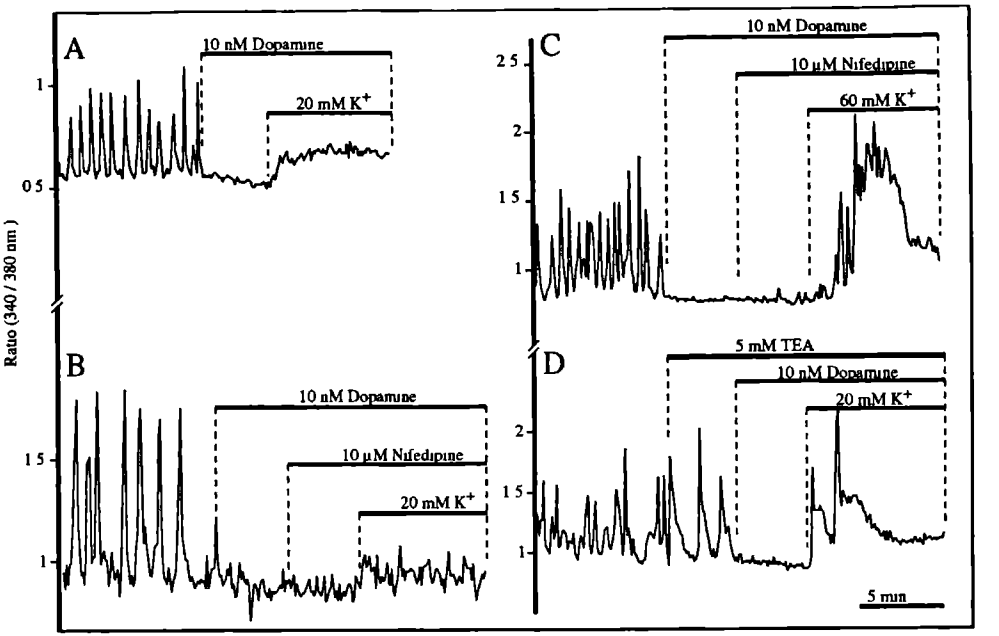


Figure 5: Effect of different $[K^+]_e$ on dopamine-inhibited melanotropes **A**, Inhibition of oscillations in all melanotropes was achieved with 10 nM dopamine Treatment of dopamine-inhibited cells with 20 mM K^+ led to a moderate, sustained increase in baseline $[Ca^{2+}]_i$ No oscillation activity was observed. **B**, 10 µM nifedipine prevented the 20 mM K^+-induced increase of baseline $[Ca^{2+}]_i$ in dopamine-inhibited cells **C**, Increasing $[K^+]_e$ from 2 mM to 60 mM in dopamine- and nifedipine-pretreated cells led to a transient increase in $[Ca^{2+}]_i$ with a fast decline phase During the first 3 min of K^+-treatment Ca^{2+} spikes were observed on top of the Ca^{2+} transient **D**, Dopamine still inhibits oscillations in TEA-pretreated cells. Subsequent addition of 20 mM K^+ led to a rapid increase in baseline $[Ca^{2+}]_i$ on top of which two Ca^{2+} spikes were observed

Neuropeptide Y

Inhibition of oscillations was achieved with 2 nM NPY. Subsequent depolarization with 20 mM K^+ increased baseline $[Ca^{2+}]_i$ in all cells (Fig. 6A, N=65). However, in about 50 % of these cells Ca^{2+} oscillations reappeared upon increasing the medium $[K^+]$ (Fig. 6B). Pretreatment with TEA had no effect on the 20 mM K^+-induced response in NPY inhibited cells, in that again about 50 % of the cells did not show Ca^{2+} oscillations during K^+-treatment (Fig. 6C, N=18). Treatment with 10 µM nifedipine during NPY-treatment completely prevented the 20 mM K^+-induced increase in baseline $[Ca^{2+}]_i$ (Fig. 6D, N=38).

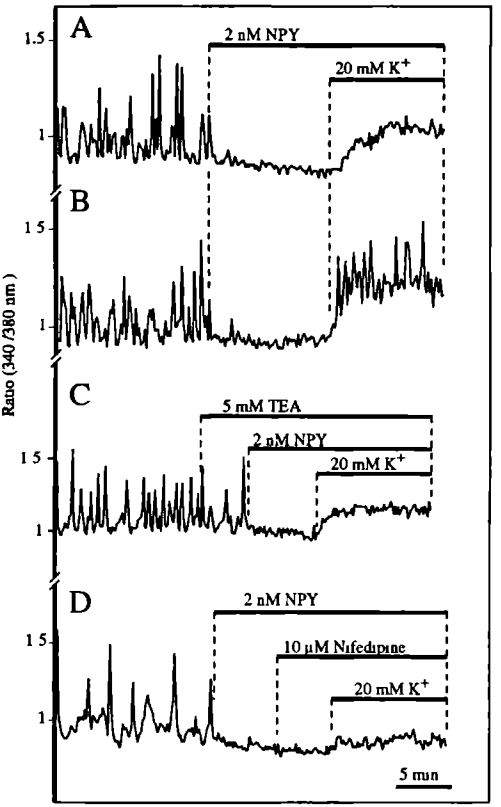


Figure 6: Effect of 20 mM K^+-treatment on NPY-inhibited cells. **A**, Ca^{2+} oscillations were inhibited by 2 nM NPY. 20 mM K^+ induced a moderate, sustained increase in baseline $[Ca^{2+}]_i$ in cells inhibited with NPY. This behavior was observed in about 50 % of the melanotropes **B**, In the remaining 50 % of the cells Ca^{2+} oscillations were observed on top of the increased baseline $[Ca^{2+}]_i$ during K^+-treatment **C**, NPY still inhibits Ca^{2+} oscillations in TEA- treated melanotropes Addition of 20 mM K^+ to these cells induced a moderate increase in baseline Ca^{2+} but no oscillation activity was observed in about 50 % of the cells which is similar to non-TEA treated cells **D**, Treatment with 10 µM nifedipine during NPY-treatment virtually completely prevented the 20 mM K^+-induced increase in baseline $[Ca^{2+}]_i$.

DISCUSSION

We have investigated the significance of the membrane potential in the control of intracellular Ca^{2+} dynamics by the secreto-inhibitors GABA, dopamine and NPY. For this purpose the effects of the inhibitors on Ca^{2+} oscillations and baseline $[Ca^{2+}]_i$ were studied in *Xenopus* melanotrope cells that were depolarized to different states by challenging them with different $[K^+]_e$.

Involvement of HVA Ca^{2+} channels in intracellular Ca^{2+} dynamics

Xenopus melanotropes possess two types of HVA Ca^{2+} channels, the N-type channel, which is sensitive to ω-conotoxin GVIA and the L-type channel, which is blocked by nifedipine [5]. It has previously been shown that the N-type channel is responsible for the occurrence of spontaneous Ca^{2+} oscillations in this cell type [6]. The present study shows that a 20 mM K^+-induced depolarization results in a moderate, sustained increase in baseline $[Ca^{2+}]_i$ and a temporary increase in oscillation frequency, results that are in agreement with earlier studies

on *Xenopus* melanotropes [19]. The oscillations were blocked by ω-conotoxin, an observation that demonstrates that the Ca^{2+} oscillations, still observed during 20 mM K^+-treatment, are brought about by the action of N-type channels. The sustained increase in baseline $[Ca^{2+}]_i$ was for the most part inhibited by nifedipine, indicating that this increase is largely caused by the action of L-type channels. Clearly, at a moderate depolarization state (20 mM K^+) both N-type and L-type channels are activated. Apparently, the N-type channels open and close in such a way that Ca^{2+} oscillations are still evoked. The fact that the frequency of the oscillations under K^+-treatment is initially higher than that of the spontaneously occurring oscillations may be due to a temporary decreased time interval between opening of the N-type channels.

Increasing $[K^+]_e$ to 60 mM results in an instantaneous rapid rise in $[Ca^{2+}]_i$ followed by a more gradual decline to an elevated plateau. Establishment of the sustained elevated plateau of this 'transient' response is completely prevented by nifedipine, demonstrating that during the late phase of the response Ca^{2+} enters entirely through L-type channels. The fact that the first part of the transient is partially blocked by ω-conotoxin demonstrates that the N-type channel is largely responsible for this rising phase of the transient.

With respect to the activation kinetics of N-type and L-type channels it is noteworthy that K^+-treatment leads to a transient activation of the N-type channel, whereas activation of L-type channels is sustained during the K^+-treatment. This transient activation of N-type Ca^{2+} channels is also observed when cells inhibited by the various secreto-inhibitors are challenged with high $[K^+]$. A possible explanation for the difference between the N- and L-type channel responses is a difference in regulation of the channels by intracellular signaling mechanisms [20]. It has been reported that in sympathic neurons the opening of the N-type channel can be modulated by association with G-proteins. According to this so-called "willing-to-reluctant transition" model, depolarization will lead to dissociation of the channel subunits from the regulatory G_o-proteins, thereby causing activation of the channel [20]. After a short period of facilitation the modulatory units would bind again resulting into channel inactivation. Alternatively, various types of G-proteins and second messengers might modulate the activity of the N-type channel [20,21].

Effects of secreto-inhibitors in relation to K^+-induced depolarizations

$GABA_A$ receptor

This study shows that the inhibition of Ca^{2+} oscillations through $GABA_A$-receptor activation by isoguvacine can be temporarily reversed by depolarizing

cells with 60 mM K^+ but not with 20 mM K^+. We recently demonstrated that isoguvacine inhibits oscillation activity by opening picrotoxin-sensitive Cl^--channels [4]. Therefore, most likely, isoguvacine inhibits oscillations by hyperpolarizing the melanotropes by stimulating influx of Cl^- through opening of Cl^--channels. The fact that the gradual increase in baseline $[Ca^{2+}]_i$ was largely sensitive to nifedipine indicates that it is due to an influx of Ca^{2+} through L-type channels. The selective action of the activated $GABA_A$-receptor on the N-type channel might indicate that the $GABA_A$ chloride channels and N-type channels are closely associated. Apparently, a 60 mM K^+-induced depolarization but not a 20 mM K^+-induced depolarization is strong enough to temporarily overcome the inhibitory effect of the isoguvacine-induced hyperpolarization with respect to N-type channel activation.

$GABA_B$ receptor

During baclofen inhibition a temporary reappearance of the oscillations was seen after a 20 mM K^+-induced depolarization. This observation can be explained by assuming that baclofen either lowers the membrane potential or increases the N-type channel threshold potential to such an extent that it is not exceeded by the spontaneous membrane depolarizations. The fact that the K^+ channel blocker TEA does not influence the baclofen-induced inhibition of oscillations strongly suggests that baclofen does not inhibit the Ca^{2+} oscillations by opening K^+-channels. Although this result does not exclude the possibility that the $GABA_B$ receptor inhibits oscillations through membrane hyperpolarization, another mechanism seems to be more likely. It has been demonstrated that activation of $GABA_B$ receptors in *Xenopus* melanotropes leads to an inhibition of the adenylyl cyclase system [13]. Since voltage-operated Ca^{2+} channels possess several phosphorylation sites which are believed to be associated with their opening [22], lowering of c-AMP levels and subsequent reduction of PKA-induced phosphorylation may cause a reduced open probability state of the N-type Ca^{2+} channel. For *Xenopus* melanotropes it has recently been shown that inhibition of the PKA activity eventually results in abolishment of Ca^{2+} oscillations [23]. Therefore, we propose that the c-AMP pathway is the main mechanism by which the $GABA_B$-receptor inhibits Ca^{2+} oscillations.

Dopamine receptor

The inhibition achieved by dopamine receptor activation is similar to the action of isoguvacine in that only a very strong depolarizing pulse of K^+ (60 mM) could induce oscillations in dopamine-treated cells. For *Xenopus* melanotropes it has been demonstrated that activation of the dopamine D_2-receptor, like the $GABA_B$-receptor, inhibits c-AMP production [13]. For other cell types, including the melanotropes of the rat and the amphibian *Rana ridibunda*, a direct coupling of the dopamine receptor with a voltage-activated K^+ channel through a G-

protein has been proposed [24-32, but see 33]. We now show a coupling of the dopamine receptor with a TEA-sensitive K^+ channel for *Xenopus* melanotropes, since addition of TEA partially reversed the inhibitory action of dopamine on the induction of oscillations by a depolarizing K^+-treatment. The observation that dopamine was still able to inhibit Ca^{2+} oscillations in the presence of TEA indicates that dopamine-induced inhibition of Ca^{2+} oscillations does not exclusively occur through K^+ channel opening. Besides inhibition through plasma membrane hyperpolarization by K^+ channel opening, dopamine might also inhibit Ca^{2+} oscillations through its inhibitory action on adenylyl cyclase. The fact that dopamine-inhibition of the K^+ depolarization was only partially reversed by TEA application supports this hypothesis.

NPY receptor

The inhibitory action of NPY more closely resembled that of baclofen than that of dopamine. While only 50 % of the cells displayed oscillations during 20 mM K^+ treatment, the NPY-induced inhibition was insensitive to TEA, indicating that NPY does not inhibit opening of the N-type Ca^{2+} channel by hyperpolarizing the cells via opening of a TEA-sensitive K^+-channel. In melanotropes of *Rana ridibunda* NPY-receptor activation leads to a complex inhibitory mechanism, including opening of voltage-operated K^+ channels and a G_o-dependent inhibition of Ca^{2+} channels [34]. For many cell types, including *Xenopus* melanotropes, it has been shown that activation of a NPY-receptor leads to an inhibition of c-AMP production through a G_i protein [4,11,35,36]. The difference in action between NPY and baclofen in *Xenopus* melanotropes might reflect the strength of inhibition on adenylyl cyclase activity and thus the phosphorylation state of the N-type channel. Alternatively, NPY may activate a G-protein capable of binding directly with the N-type channel, as recently reported for sympathetic neurons [20,37]. It is interesting that the NPY-induced inhibition leads to a heterogeneous cellular response with only 50 % of the cells giving Ca^{2+} oscillations after K^+-induced depolarization. A possible explanation for this cellular heterogeneity might be a difference in the susceptibility of the N-type channels to G-protein binding in individual melanotropes.

Conclusions

Based on the observations presented in this paper it can be concluded that the different secreto-inhibitors inhibit the occurrence of spontaneous Ca^{2+} oscillations in *Xenopus* melanotropes in different ways. Whereas baclofen most likely inhibits the oscillations by dephosphorylating the N-type channel, dopamine causes additionally a hyperpolarization by opening of a K^+-channel. Although NPY-induced inhibition is stronger than that of baclofen, no involvement of a TEA-sensitive K^+ channel could be demonstrated. The

inhibition by isoguvacine most likely reflects a hyperpolarization by opening of Cl^--channels. Finally, the present study demonstrates that the main site of action of the secreto-inhibitors is on the N-type channel and not on the L-type channel, since a K^+-induced depolarization in all cases leads to a nifedipine-sensitive elevation of the baseline Ca^{2+} values in cells treated with the various secreto-inhibitors. While the N-type channel is clearly involved in regulation of the secretory process in *Xenopus* melanotropes [3, 4], the functional significance of the L-type channel remains to be established.

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SPATIAL AND TEMPORAL ASPECTS OF CA^{2+} OSCILLATIONS IN *XENOPUS LAEVIS* MELANOTROPE CELLS

With Bruce G Jenks, Renier J.A M. van Dinter and Eric W. Roubos

The spatiotemporal aspects of Ca^{2+} signaling in melanotrope cells of *Xenopus laevis* have been studied with confocal laserscanning microscopy. In the whole-frame scanning mode two major intracellular Ca^{2+} compartments, the cytoplasm and the nucleus, were visualized. The basal [Ca^{2+}] in the nucleus appeared to be lower than that in the cytoplasm and Ca^{2+} oscillations appear to occur synchronously in both compartments. Upon addition of the N-type channel blocker ω-conotoxin oscillations in both regions disappeared, indicating a strong coupling between the two compartments with respect to Ca^{2+} dynamics. In the line-scanning mode, yielding a higher time-resolution, it was found that the rise phase of a Ca^{2+} oscillation is not a continuous process but consists of three or four discrete steps. Each step can be seen as a Ca^{2+}-wave, starting at the cell membrane going through the cytoplasm at a speed of 33.3 ± 4.3 μm.s^{-1}. Before the Ca^{2+}-wave enters the nucleus a delay of 120.0 ± 24.1 ms occurred. In the nucleus the speed of a wave was 80.0 ± 3.0 μm.s^{-1}. Treatment with the Ca^{2+}-ATPase inhibitor thapsigargin (1 μM) almost completely eliminated the apparent difference in the basal [Ca^{2+}] in the cytoplasm and the nucleus, reduced the delay of a Ca^{2+}-wave before entering the nucleus to 79.8 ± 8.7 ms, and diminished the nuclear wave speed to 35.0 ± 4.9 μm.s^{-1}. These results indicate that a cytoplasmic thapsigargin-sensitive ATPase near the nuclear envelope may be involved in buffering Ca^{2+} before entering the nucleus. After sensitizing IP_3 receptors by thimerosal (10 μM) the speed of the cytoplasmic Ca^{2+}-wave was increased to 70.3 ± 3.6 μm.s-1, strongly suggesting that IP_3 receptors are involved in the propagation of the cytoplasmic Ca^{2+} wave. Our results indicate that, in melanotropes the generation and propagation of Ca^{2+} oscillations is a complex event, involving influx of Ca^{2+} through N-type Ca^{2+} channels, propagation of the cytoplasmic Ca^{2+} wave through IP_3-sensitive intracellular stores and thapsigargin-sensitive Ca^{2+}-uptake mechanisms, and a regulated Ca^{2+} entry into the nucleus.

In melanotrope cells of the amphibian *Xenopus laevis* spontaneous Ca^{2+} oscillations have been described [1-4]. These cells secrete α-melanophore-stimulating hormone (α-MSH) causing darkening of the skin during adaptation of the animal to a dark background [5]. Secretion of α-MSH depends on Ca^{2+} influx through voltage-operated N-type Ca^{2+} channels [6]. It has also been shown that Ca^{2+} oscillations in these cells depend on influx of extracellular Ca^{2+} [4] through opening of N-type Ca^{2+} channels [1]. Moreover, neural factors that inhibit α-MSH secretion also inhibit Ca $^{2+}$ oscillations whereas secretostimulators induce these oscillations [2,3]. Therefore, it has been hypothesized that Ca^{2+} oscillations are the driving force for hormone secretion [2,3,7]. Although Ca^{2+} signaling in *Xenopus* melanotropes has been studied in some detail with respect to membrane channel opening, little is known about the intracellular (cytoplasmic and nuclear) dynamics of these oscillations.

The last few years the development of high-resolution Ca^{2+} imaging techniques was paralleled by an increased interest in spatiotemporal aspects of Ca^{2+} signaling to see how Ca^{2+} signals are propagated in a cell. For many cell types it has been shown that changes in [Ca^{2+}] (spikes and oscillations) occur in both the cytoplasm and the nucleus [8-14]. Propagation of a Ca^{2+} wave through

the cytoplasm can occur through IP_3 and/or ryanodine-sensitive Ca^{2+}-induced Ca^{2+}-release (CICR) or through diffusion [15]. However, little is known about the propagation of Ca^{2+} oscillations in the nucleus. For neuronal cell lines it has been proposed that an increase in the nuclear $[Ca^{2+}]$ is a process driven by passive diffusion [8] whereas in several smooth muscle cells an active component in nuclear Ca^{2+} oscillations has been proposed [9,10]. To gain further insight into spatiotemporal aspects of Ca^{2+} oscillations in *Xenopus* melanotropes Ca^{2+} dynamics were studied, using confocal laserscanning microscopy on isolated cells loaded with visual wavelength fluorescent Ca^{2+} indicators. Special attention has been paid to the dynamics of the Ca^{2+} oscillations in time (temporal aspect) and to the distribution of these oscillations in cytoplasm and nucleus (spatial aspect). The present study shows that Ca^{2+} oscillations in *Xenopus* melanotropes are complex signals which start with the opening of N-type Ca^{2+} channels at the plasma membrane; the resulting oscillations are propagated through the cytoplasm as Ca^{2+} waves, a process in which most likely IP_3 receptors are involved. An oscillation rise phase consists of 3 or 4 discrete steps. After a delay an oscillation enters the nucleus. The delay is a controlled event, probably involving thapsigargin-sensitive Ca^{2+}-ATPases.

MATERIALS AND METHODS

Animals

Young-adult *Xenopus laevis* were taken from laboratory stock and kept on a black background for three weeks under continuous illumination, at 22 °C The animals were fed weekly with beef heart

Preparation of single cells

Isolation of melanotrope cells was performed as described previously [1,2] In short, after anesthetization in a MS222 solution (1 g/l, Sigma, St Louis, MO, USA) animals were perfused with *Xenopus* Ringer's solution, containing 112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$ and 15 mM Ultral-HEPES (Calbiochem, La Jolla CA, USA, pH 7 4), to remove blood cells Then neurointermediate pituitary lobes were dissected and incubated for 45 min in Ringer's solution without $CaCl_2$ to which 0 25 % (w/v) trypsin (Gibco, Renfrewshore, UK) had been added Cells were subsequently dispersed in Leibovitz's L15 medium, containing 10 % fetal calf serum (Gibco), by gentle trituration of the lobes with a siliconized Pasteur's pipette The medium had been adjusted to *Xenopus* blood osmolality (L15 ultrapure water = 2 1) After washing, cells were plated on glass cover slips coated with poly-L-lysine (Mw > 300 kD, Sigma) at a density of about 10,000 cells/slip, and cultured for 3 days at 22 °C The cells were readily identified on the basis of their characteristic round shape

$[Ca^{2+}]$ measurements

In order to determine possible differences in the relative Ca^{2+} concentrations in the cytoplasm and the nucleus, coverslips were placed in a Leiden chamber [16] and melanotropes were loaded with the two visible wavelength Ca^{2+}-indicators fluo-3 and fura-red (4 μM and 6

μM, respectively, Molecular Probes, Eugene, Or, USA) in the presence of 1 μM pluronic F-127 [17] (Molecular Probes) for 25 min at 20 °C For time-related measurements of changes in fluorescence intensity, cells were loaded with 6 μM fura-red in the presence of 1 μM pluronic F-127 for 25 min at 20 °C Then, cells were washed 3 times with medium and were allowed to equilibrate for 30 min Prior to measurements cells were placed on an Nikon Diaphot microscope (Nikon, Tokyo, Japan) equipped with a 60x oil-immersion objective (N A = 1 4) Confocal laserscanning microscopy (CLSM) was performed on a BioRad MRC-600 (BioRad, Hemel Hempstead, UK) Excitation at 488 nm was obtained by directing the light of a 15 mW Ar-laser through an excitation filter (488 DF 10) To reduce bleaching of the fluorescent probes laser emission was attenuated to about 10% by introducing a neutral density filter in front of the laser Fluorescent light was passed through a dichroic reflector (DR 510 LP) and a barrier filter (OG 515) Dual-emission measurements were performed via two photo-multiplier tubes (PMT) by directing the fluorescence light through a dichroic reflector (DR 565 LP) Fluo-3 fluorescence was collected in a PMT in front of which a bandpass filter (540 DF 30) was placed Fura-red emission was collected simultaneously in the second PMT in front of which a long-pass filter (EF 600 LP) had been placed Optical sections of approximately 1 μm were obtained by opening the confocal pinholes in front of the PMT's for 50% On-line measurements of fluorescence intensity, utilizing the fast photon counting setup of the MRC-600, were obtained from pre-selected areas of interest using the time-course ratiometric software package (TCSM, BioRad) Changes in intracellular Ca^{2+} are expressed as changes in fluorescence intensity During whole-frame scanning the scan frame was sized at 192x128 pixels, allowing a scan time of 0 45 s per frame In experiments where line-scanning was performed, the frame size was 384x256 pixels and the scan-time per line was 6 ms The scanning line was set in such a way that it transsected the midline of a melanotrope cell Four such lines were averaged to obtain a better signal-to-noise ratio, leading to a final time resolution of 24 ms Analyzing melanotropes in this way allowed scanning of one frame within 1 5 s

Nuclear staining

After Ca^{2+} experiments were performed, cells were fixed on stage in Bouins' fixative for 15 min, after which they were washed three times with *Xenopus* Ringers' solution and incubated with 10 μg/ml propidium iodine (Sigma) for 10 min After this staining cells were washed two times with *Xenopus* Ringers' solution, and studied with the CLSM (excitation at 514 nm, emission wavelength > 550 nm)

Calculations

In order to determine the time at which a Ca^{2+} wave started at each measure point, a fast-Fourier transformation [18] was carried out on data obtained from line-scanning experiments The transformed data points were subjected to a CUSUM-test [19], the intersection of the CUSUM-data with the X-axis was taken as the starting point of a Ca^{2+} change for that specific region

RESULTS

In the present study Ca^{2+} oscillations in melanotrope cells were monitored using CLSM. Approximately 80 % of the cells displayed spontaneous oscillations, consisting of individual Ca^{2+} spikes arising from the basal [Ca^{2+}]. The increase period of a spike is referred to as the rise phase (see Fig. 1,2). The frequency of the oscillations proved to be remarkably constant for any given cell. The relative amplitude, as determined by comparing the maximum spike value with the basal fluorescence was constant for each spike for any given area within a cell (see e.g. Fig. 2).

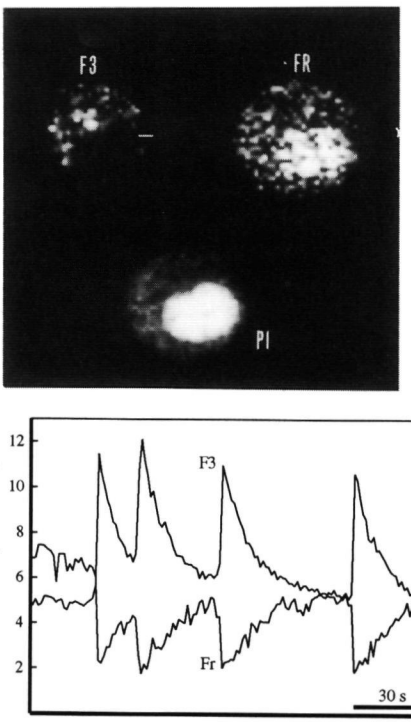

Figure 1: Loading properties of fluo-3 and fura-red in *Xenopus* melanotropes **A**, Whole-frame scanning of a melanotrope loaded with fluo-3 (F3) and fura-red (Fr). After imaging the Ca^{2+} dynamics this cell was fixated on stage and a nuclear stain with propidium iodine (PI) was performed. **B**, Whole frame scanning of a spontaneously oscillating melanotrope with a time-interval of 2 s between frames. At the time of a Ca^{2+} oscillation the Fluo-3 intensity increased and simultaneously the Fura-red intensity decreased.

Spatial aspects of Ca^{2+} oscillations

Comparing the distribution of fluorescence intensity of both Ca^{2+}- sensitive indicators fluo-3 and fura-red with a nuclear staining, a clear compartmentalization of the Ca^{2+} signal was observed (Fig. 1A, N=6). The fluo-3 fluorescence intensity was lower in the nucleus (Fn) than in the cytoplasm (Fc) (Fn/Fc = 0.8 ± 0.1) whereas the fura-red signal was highest in the nucleus (Fn/Fc = 1.2 ± 0.1). In oscillating melanotropes the changes in fluorescence intensity of the two probes in time had opposite directions (Fig. 1B, N=6). At the start of the experiment the basal fluorescence intensities of fluo-3 and fura-red were similar

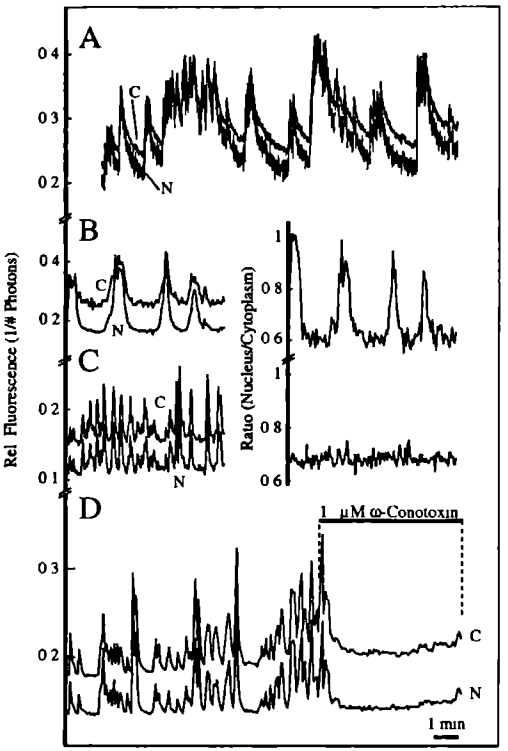

Figure 2: Whole-frame scanning experiments of *Xenopus* melanotropes **A**, Whole frame scanning of a spontaneous oscillating melanotrope with a time interval of 0 45 s/frame The reciprocal values of the Fura-red intensity for a cytoplasmic and nuclear region are presented Oscillations in cytosol and nucleus appeared to start at the same time point **B, C**, Several melanotropes were monitored at the same time with a time interval of 2 s Shown are the reciprocal values of the Fura-red fluorescence intensity of a cytoplasmic and nuclear region of two cells In 60 % of the cells the changes in the nuclear fluorescence intensity were in harmony with those in the cytosol, leading to a stable ratio Fn/Fc (**C**) In the remaining 40 % of the cells the ratio Fn/Fc changed due to a stronger fluorescence change in the nucleus (**B**) **D**, Both cytoplasmic and nuclear Ca^{2+} oscillations are sensitive to ω-conotoxin GVIA ω-Conotoxin was added to the bathing solution by gentle pipetting, yielding a final concentration of 1 µM

but their bleaching ratio's differed: at a sampling rate of 1 image every 2 s, fluo-3 decreased in fluorescence intensity by 6 % per min and fura-red by less than 1 % per min (Data not shown).

In order to follow the $[Ca^{2+}]$ dynamics in cytoplasm and nucleus in time, changes in fluorescence intensity of fura-red were monitored. Performing whole frame scanning with a time resolution of 0.45 s, Ca^{2+} oscillations in cytoplasm and nucleus seemed to occur synchronously (Fig. 2A, N=5). In 60 % of the cells the relative amplitude of the nuclear oscillations remained similar to that in the cytoplasm, leading to a stable fluorescence ratio (nucleus/cytoplasm) (Figure 2C, N=22), while in the other 40 % the relative amplitude of Ca^{2+} oscillations in the nucleus increased compared to the cytoplasm, leading to an increase in fluorescence ratio (Fig. 2B, N=14). Oscillations in both cytoplasm and nucleus were fully inhibited by 1 µM ω-conotoxin GVIA (Fig. 2D, N=13).

Observing Ca^{2+} oscillations with the line-scanning mode (Fig. 3A, N=8) showed that increases in $[Ca^{2+}]$ start at the cell membrane and form a Ca^{2+} wave

that spreads concentrically. The average speed of the wave in the cytoplasm is 33.3 ± 4.3 μm.s^{-1}. The speed variation between individual cells is between 25 and 40 μm.s^{-1} but the speed of a Ca^{2+} wave is very constant for any given cell. At the nuclear membrane an apparent delay of 120.0 ± 24.1 msec was observed before the Ca^{2+}-wave entered the nucleus (Fig. 3B). The nuclear speed of a wave was 80.0 ± 3.0 μm.s^{-1}. The rise phase of one wave was 300 msec whereas the

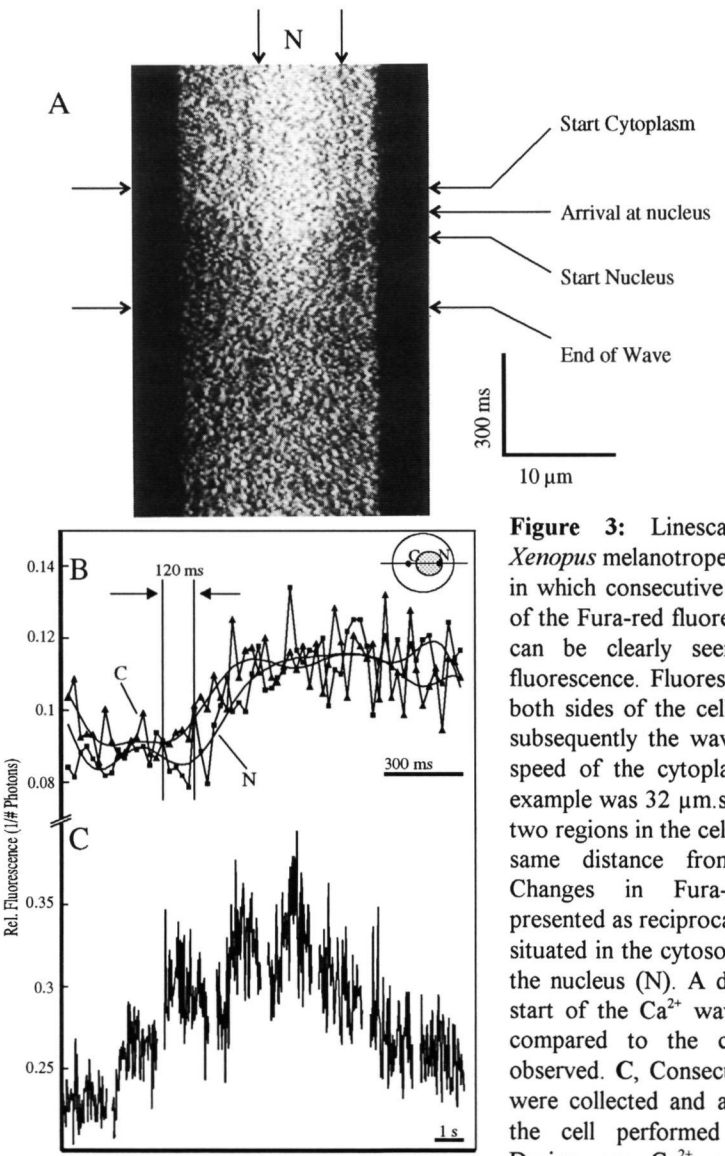

Figure 3: Linescanning experiments on *Xenopus* melanotropes. **A**, Example of a frame in which consecutive linescans were collected of the Fura-red fluorescence. The nucleus (N) can be clearly seen as a band of high fluorescence. Fluorescence changes started at both sides of the cell membrane (arrow) and subsequently the wave spreaded inward. The speed of the cytoplasmic Ca^{2+} wave in this example was 32 μm.s^{-1}. **B**, On-line analysis of two regions in the cell (presented in 2A) at the same distance from the cell membrane. Changes in Fura-red fluorescence are presented as reciprocal values. One region was situated in the cytosol (C), the other region in the nucleus (N). A delay of 180 msec in the start of the Ca^{2+} wave at the nuclear region compared to the cytoplasmic region was observed. **C**, Consecutive frames of linescans were collected and analysis of one region in the cell performed after the experiment. During one Ca^{2+} oscillation 4 consecutive waves were observed.

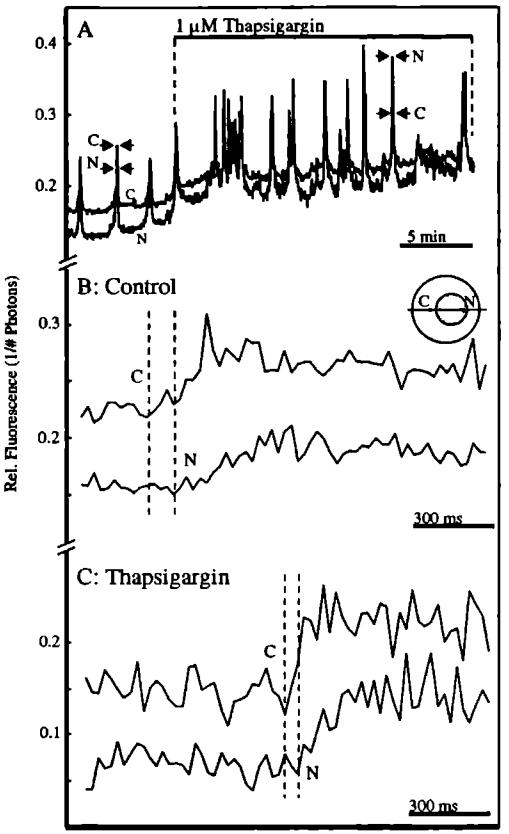

Figure 4: Involvement of thapsigargin-sensitive Ca^{2+} stores. In all experiments thapsigargin was gently pipetted into the bathing solution, to a final concentration of 1 µM. **A**, Whole-frame recording of a spontaneous oscillating melanotrope cell. Shown are the reciprocal values of the fura-red fluorescence of a cytoplasmic (C) and nuclear (N) region. After thapsigargin treatment the Ca^{2+} signal in both compartments increased and the fluorescence signals gradually converged. Indicated are the peak-heights of an oscillation during normal and thapsigargin-treatment (arrows) **B**, Linescanning experiment. Shown are reciprocals of fura-red fluorescence of two regions at the same distance from the cell membrane, one region situated in the cytosol (C) and the other in the nucleus (N). The delay of the Ca^{2+} wave to enter the nucleus for this cell was 120 msec (dotted lines). **C**, Linescanning of the same cell as in **B** at the same position after 15 min thapsigargin treatment. The delay of the Ca^{2+} wave to enter the nucleus was 48 msec.

rise phase of a Ca^{2+} spike, as observed with whole-frame analysis, varied between 5 and 20 s (Fig. 2B,D). Consecutive frames of line scans showed that each Ca^{2+} spike consisted of three to four distinct Ca^{2+} waves, seen as discrete steps of Ca^{2+} increase at each point in the cell (Fig. 3C, N=7). Each individual Ca^{2+} wave also entered the nucleus, as is illustrated in Fig. 3A.

Involvement of intracellular Ca^{2+}-stores in Ca^{2+} oscillations

1 µM thapsigargin converged the fura-red fluorescence intensity in cytoplasm and nucleus within 15 min after application (Fig. 4A, N=15). In control situations the relative amplitude of the oscillations in the nucleus was always lower than in the cytoplasm (Fig. 4A). During thapsigargin treatment the relative amplitude of the nuclear oscillations became higher than in the cytoplasm (Fig. 4A). After 15 min thapsigargin treatment a reduction of the delay at the nuclear envelope from 120.0 ± 24.1 msec to 79.8 ± 8.7 msec was observed (Fig.

4B *versus* Fig. 4C, N=5). Moreover, the speed of the Ca^{2+} wave in the nucleus was reduced from 80.0 ± 3.0 μm.s^{-1} to 35.0 ± 4.9 μm.s^{-1}.

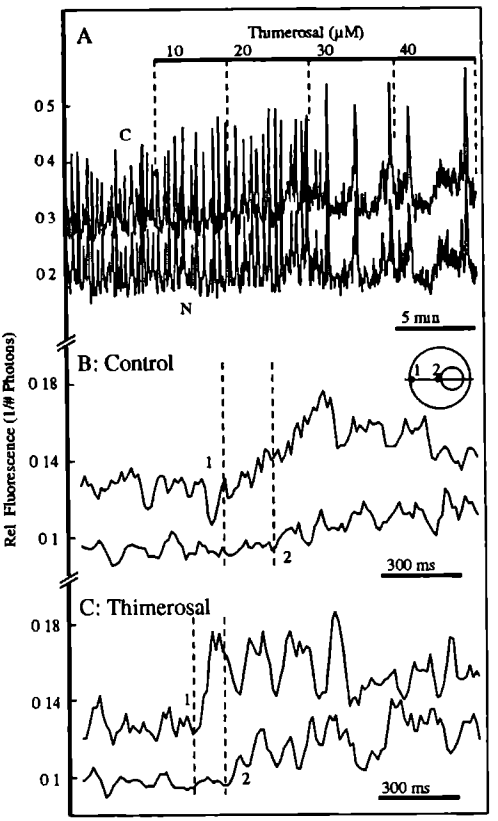

Figure 5: Involvement of thimerosal-sensitive IP_3 receptors in Ca^{2+} oscillations in *Xenopus* melanotropes. **A**, Whole-frame scanning experiment. Per experiment 7 melanotropes were monitored on-line Thimerosal was gently pipetted in the bathing solution to reach the final concentrations indicated in the graph Thimerosal (10 and 20 μM) had no effect on the frequency of spontaneous oscillating melanotropes Increasing the concentration to 30 μM led to a drastic decrease in frequency **B**, Linescanning experiment with an oscillating cell. Shown are reciprocals of fura-red fluorescence of two regions, one situated in near the cell membrane (1) and a second region 7 μm deeper into the cytosol (2) close to the nucleus The speed of the cytoplasmic Ca^{2+} wave for this cell was 38 μm s^{-1}. Dotted lines indicate the starting point of the Ca^{2+} increase for that point. **C**, Linescanning of the same cell at the same position after addition of 10 μM thimerosal The speed of the cytoplasmic Ca^{2+} wave was increased to 70 μm s^{-1}.

Thimerosal (10 or 20 μM), a thiol reagent that, when applied at low concentrations, sensitizes IP_3 receptors [20], had no effect on the frequency (Fig. 5A, N=20) nor on the relative amplitude of the Ca^{2+} oscillations. Increasing the concentration to 30 μM drastically decreased the frequency of the oscillations (Fig. 5A). Imaging melanotropes with the linescanning mode showed that application of 10 μM thimerosal increased the speed of the cytoplasmic Ca^{2+} wave from 33.3 ± 4.3 μm.s^{-1} to 70.3 ± 3.6 μm.s^{-1} (Fig. 5B *versus* C, N=4); thimerosal had no effect on the speed of the nuclear Ca^{2+} wave nor on the delay observed at the nuclear envelope (Data not shown).

DISCUSSION

In the present study we investigated spatio-temporal aspects of spontaneous Ca^{2+} oscillations in melanotrope cells of *Xenopus laevis* using CLSM. It has previously been found that Ca^{2+} oscillations in these cells arise from Ca^{2+} entry and that blocking voltage-operated Ca^{2+} channels during any time of the rise phase of a Ca^{2+} spike stopped the oscillations [4]. Subsequent studies showed that the Ca^{2+} influx came through opening of voltage-operated N-type Ca^{2+} channels [1]. All studies in *Xenopus* melanotropes have so far been concentrated on Ca^{2+} entry mechanism and little is known about the intracellular aspects of these Ca^{2+} oscillations.

Loading melanotropes with both fluo-3 and fura-red showed that the fluorescence intensities of these probes in the nucleus were different from those in the cytoplasm: for fluo-3 the fluorescence was less intense in the nucleus than in the cytoplasm whereas fura-red showed a reverse picture. Therefore, these results strongly suggest that the $[Ca^{2+}]$ in the nucleus is lower than in the cytoplasm although it can not be excluded that the differences in fluorescence intensities found are due to different loading capacities of the nucleus compared to cytoplasm for each probe. We show that changes in fluo-3 and fura-red fluorescence intensities during time occur in opposite directions, thus demonstrating that these changes reflect changes in $[Ca^{2+}]$. It is known that fluo-3 has a higher fluorescence intensity when bound to Ca^{2+} [21-23], whereas fura-red has a lower fluorescence intensity when bound to Ca^{2+}. For myocytes a calibration method for Ca^{2+} has been described based on a double loading with fluo-3 and fura-red. However, use of this calibration technique is impossible for *Xenopus* melanotropes because the bleaching properties of the two probes differ. Therefore, in experiments with a long time-course and with intensive laser-scanning we consider the fura-red signal to be the most reliable as it bleaches less than 1 % per min.

Two facts indicate that oscillations in the cytoplasm and nucleus arise from the same oscillator. First, even at the highest scanning frequency possible in whole-frame mode (0.45 s/frame) oscillations in cytoplasm and nucleus appeared at the same time and had the same duration. Second, both oscillations in cytoplasm and nucleus were inhibited by ω-conotoxin. The latter observation indicates that the generation of the oscillations takes place at the plasma membrane through opening of N-type channels. The only difference between cytoplasmic and nuclear oscillations was seen when melanotropes were studied in the line-scanning mode. Increasing the time resolution to 24 ms showed that the oscillation concentrically moves inwards from the membrane into the cell as a Ca^{2+} wave. From comparing oscillations in cytoplasmic and nuclear regions at the same distance from the plasma membrane it appeared that a cytoplasmic Ca^{2+}

wave undergoes a delay of approximately 120 ms before entering the nucleus. Also for RBL cells such a time delay was found and it was hypothesized that changes in the nuclear [Ca^{2+}] passively follow that in the cytoplasm [13]. However, in *Xenopus* melanotropes the fluorescence intensities in cytoplasm and nucleus converge after treatment with thapsigargin. Moreover, the delay in the Ca^{2+} signal entering the nucleus is substantially shortened by thapsigargin. The decrease in the convergence fluorescence intensities as well as in time delay in the cytoplasm and the nucleus after thapsigargin treatment can be explained by assuming that Ca^{2+}-ATPase activity in the cytoplasm or at the nuclear envelope, reduces the amplitude of a Ca^{2+} oscillation, as it passes through the cell. In this way intracellular Ca^{2+}-ATPase activity might buffer Ca^{2+} when a Ca^{2+} wave reaches the vicinity of the nuclear envelope or is entering the nucleus. Possibly, Ca^{2+}-ATPase activity delays the moment that Ca^{2+} reaches a threshold for the signal to enter the nucleus. Such a mechanism might provide a control point through which a cell can set the amplitude of the Ca^{2+} signal entering the nuclear compartment. The location of this thapsigargin-sensitive Ca^{2+}-ATPase activity, as well as its physiological significance remains to be determined. In smooth muscle cells, an active component sensitive to thapsigargin in transporting a Ca^{2+} signal to the nucleus has also been described [9,10].

From analysis of the Ca^{2+} oscillations using whole-frame scanning it can be calculated that the rise phase lasts about 5 to 20 s. This period is similar to that shown for Ca^{2+} oscillations in *Xenopus* melanotropes using digital imaging methods [1,4]. It was therefore surprising to see that the rise phase of a Ca^{2+} wave lasts only 300 ms. This seeming discrepancy was resolved when consecutive frames of linescanning were taken, showing that each Ca^{2+} oscillation consists of 3 to 4 consecutive Ca^{2+} waves occurring over a period of 5-20 s. This is the first report of a stepwise increase in intracellular Ca^{2+} for secretory cells. For pancreatic acinar cells it has been described that the increase in [Ca^{2+}] during a Ca^{2+} oscillations is a continuous process involving three intracellular regions with different sensitivities to ryanodine and IP_3 [15,24,25]. There are at least two possible mechanisms for the stepwise increase in Ca^{2+} in *Xenopus* melanotropes. First, the stepwise increase in Ca^{2+} might be a property of an intracellular Ca^{2+}-propagator mechanism. Second, spontaneous membrane depolarizations, observed in *Xenopus* melanotropes [6], might take place periodically, accounting for a temporary opening of the N-type Ca^{2+} channel, and thus stepwise increases in [Ca^{2+}]. Since inhibition of voltage-operated Ca^{2+} channels during the rise phase of a Ca^{2+} spike blocked the formation of this spike [4], we feel that the stepwise increase during a Ca^{2+} oscillation in *Xenopus* melanotropes is more likely due to a function of the plasma membrane depolarizations. Possibly, the number of consecutive Ca^{2+} waves in *Xenopus* melanotropes can be regulated in order to set the amplitude and duration of a Ca^{2+} spike.

The speed of a cytoplasmic Ca^{2+} wave ranged between 25 and 40 $\mu m.s^{-1}$. The diffusion radius for Ca^{2+} is less than 1 μm [26]. Therefore, it seems unlikely that the propagation of the Ca^{2+} wave is exclusively by diffusion of intracellular Ca^{2+} through the cytoplasm. According to Jaffe CICR would propagate a Ca^{2+} wave with a speed of 20 - 30 $\mu m.s^{-1}$ [27]. Such propagation can be through a ryanodine-sensitive CICR or a Ca^{2+}-sensitive IP_3-receptor [15,28]. For *Xenopus* melanotropes we have demonstrated that a ryanodine-sensitive CICR is not involved in the generation of Ca^{2+} oscillations [1]. In order to test a possible role of the Ca^{2+}-sensitive IP_3-receptor in the propagation of a Ca^{2+} wave, thimerosal was used. It has been demonstrated by Bootman that this thiol-reagent in low concentrations can increase the sensitivity of the IP_3 receptor for IP_3 and hence can increase oscillation frequency when such oscillations are sensitive to IP_3 [20]. For *Xenopus* melanotropes we show no difference in frequency or amplitude of the Ca^{2+} oscillations after application of low concentrations of (10 to 20 μM) thimerosal. Higher concentrations lead to a drastic reduction of the frequency, a result that can be explained by inhibition of all cellular ATPase activity [20]. Although no difference in frequency was observed, the speed of the Ca^{2+} waves through the cytoplasm almost doubled when melanotropes were treated with low concentrations of thimerosal, indicating the likely involvement of IP_3 sensitive stores in the propagation of the Ca^{2+} signal through the cytoplasm. In general, the Ca^{2+}-ATPase activity on the IP_3-sensitive intracellular Ca^{2+} pool is sensitive to thapsigargin [28,29]. In *Xenopus* melanotropes the presence of thapsigargin-sensitive Ca^{2+}-ATPases on IP_3 sensitive intracellular Ca^{2+} stores is not established. In order to determine such presence localization studies need to be performed.

The observed speed of a nuclear Ca^{2+} wave was approximately 80 $\mu m.s^{-1}$. For hepatocytes it has been postulated that an IP_3-sensitive Ca^{2+} pool is present in the nucleus [30]. Such a Ca^{2+} pool could not be demonstrated in *Xenopus* melanotropes since low concentrations of thimerosal had no effect on the speed of the nuclear Ca^{2+} wave.

In conclusion, these results give strong indications that for *Xenopus* melanotropes Ca^{2+} oscillations, generated by Ca^{2+} influx through voltage-sensitive N-type Ca^{2+} channels, is propagated through the cytoplasm by an IP_3-sensitive Ca^{2+}-release mechanism. The existence of such a mechanism can be of importance to the functioning of a cell, since it allows integration of signals generated at the cell membrane with several cellular processes *i.e.* the secretory process, biosynthesis or gene expression [11,14,31,32].

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GENERAL DISCUSSION

Based on the experimental data presented in this thesis, a model of Ca^{2+} dynamics in the melanotrope cell of *Xenopus laevis* is presented in Fig. 1. Central to this model is the periodic opening of the N-type Ca^{2+} channels to initiate intracellular Ca^{2+} oscillations. The occurrence of spontaneous Ca^{2+} oscillations in pituitary melanotrope cells may be a species-specific phenomenon, since it is not observed in rat melanotropes [1]. The opening of the N-type channel very likely occurs as a consequence of membrane depolarization, as revealed by the occurrence of spontaneous action potentials in the *Xenopus* melanotrope cells (Chapters 1 and 2). The oscillations probably are an important driving force for secretion, because it was found that inhibition of the N-type Ca^{2+} channel by ω-conotoxin GVIA inhibits secretion (Chapter 1). Moreover, secreto-inhibitors decreased the frequency of the oscillations but had little or no effect on baseline Ca^{2+}, indicating that baseline Ca^{2+} is not directly involved in the regulation of secretion (Chapters 3 and 4). Similarly, sauvagine, an amphibian

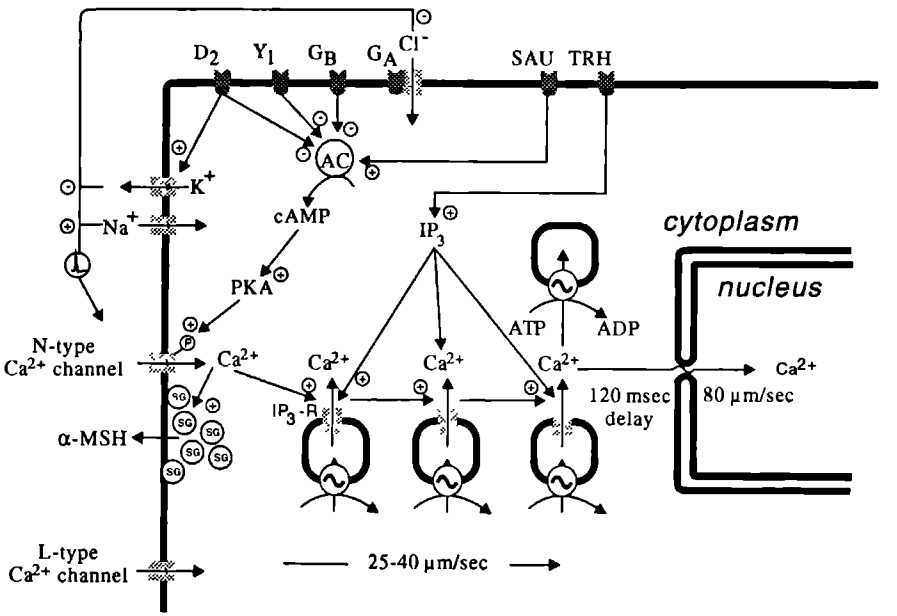

Figure 1: Model for generation and regulation of Ca^{2+} oscillations in *Xenopus* melanotropes. Abbreviations. IP_3-R: inositol-1,4,5-trisphosphate receptor, PKA cAMP-dependent protein kinase; AC: adenylyl cyclase; SG: secretory granules, α-MSH α-melanophore-stimulating hormone; TRH: thyrotropin releasing hormone receptor, SAU: savagine receptor, G_A: $GABA_A$ receptor; G_B: $GABA_B$ receptor, Y_1: NPY receptor, D_2: dopamine receptor Explanation of all the processes is described in the text.

peptide related to CRH, which stimulates secretion, caused an increase in frequency of Ca^{2+} oscillations (Chapter 3). The latter observation indicates that modulation of secretory activity can be through frequency modulation of the Ca^{2+} oscillations.

Two mechanisms would appear to be important in regulating the activity of the N-type Ca^{2+} channel in generating Ca^{2+} oscillations, namely phosphorylation through a c-AMP-dependent mechanism and membrane depolarization. The importance of c-AMP appears first from the fact that the secreto-inhibitors dopamine, NPY and baclofen have been show to inhibit c-AMP production in *Xenopus* melanotropes [2, Chapter 4] while the secreto-stimulator sauvagine stimulates c-AMP production [2]. Furthermore, it is shown that a membrane-permeable c-AMP analog increases the frequency of Ca^{2+} oscillations in oscillating cells and induces the occurrence of oscillations in non-oscillating cells [3, Chapter 4]. Finally, inhibition of c-AMP-dependent protein kinase activity in *Xenopus* melanotropes inhibits Ca^{2+} oscillations [3].

The importance of membrane depolarization in the regulation of N-type channel activity is shown by two facts. First, inhibition of oscillations and secretion by isoguvacine is achieved by opening of a chloride channel, presumably leading to an influx of Cl^- leading to hyper- polarization; consistent with this idea is the observation that a strong de- polarization with K^+ abolishes $GABA_A$ receptor-induced inhibition of Ca^{2+} oscillations (Chapters 3 and 5). Second, a depolarizing pulse of K^+ increases the frequency of oscillations in *Xenopus* melanotropes (Chapter 5). Studies using the K^+ channel blocker TEA indicated an interesting difference between the action of the $GABA_B$ and dopamine D_2 receptor mechanisms in inhibiting the Ca^{2+} channel. While the $GABA_B$ receptor mechanism would appear to work strictly through its inhibitory action on adenylyl cyclase, the D_2 receptor apparently functions not only through inhibition of production of c-AMP but also through activation of K^+ channels leading to membrane hyperpolarization (Chapter 5).

It became evident in the studies conducted with confocal laserscanning microscopy (Chapter 6) that the Ca^{2+} oscillations occur throughout the melanotrope cell. Using the linescanning mode for high temporal resolution it was shown that the oscillations are initiated at the membrane and travel towards the nucleus at a speed of 25-40 $\mu m.s^{-1}$, which is too fast to be accounted for by simple Ca^{2+} diffusion [4,5]. Since ryanodine is unable to alter the dynamics of Ca^{2+} oscillations, an involvement of a ryanodine-sensitive CICR seems unlikely (Chapter 2). However, low concentrations of thimerosal, which are known to sensitize IP_3 receptors [6], increased the speed of the cytoplasmic Ca^{2+} wave (Chapter 6). Therefore, in the model a process of Ca^{2+}-induced Ca^{2+}-release (CICR) involving IP_3 receptors and mobilization of intracellular Ca^{2+} stores has been engaged to explain the propagation of the Ca^{2+} signal to the nuclear envelope. Successive line scans showed that, in each point of the cell, the rise

phase of each Ca^{2+} oscillation consisted of 3 to 4 discrete steps of Ca^{2+} increase. Each increase step possibly reflects a spontaneous membrane depolarization, resulting in opening of the N-type channels. The subsequent propagation of the wave can be explained by assuming the presence of multiple intracellular Ca^{2+} stores with different sensitivities to $[Ca^{2+}]_i$. Linescanning also revealed a 120 ms delay before the Ca^{2+} wave entered the nucleus to be continued at a speed of 80 $\mu m.s^{-1}$. Treatment with thapsigargin decreased this delay and increased the amplitude of nuclear oscillations relative to that of cytoplasmic oscillations. Therefore it is proposed that a thapsigargin-sensitive Ca^{2+}-ATPase is involved in buffering Ca^{2+} in the vicinity of the nuclear membrane thus affecting Ca^{2+} signaling inside the nucleus. Since thapsigargin is ineffective in blocking the occurrence of cytoplasmic Ca^{2+} oscillations, two intracellular stores have been implemented; one sensitive to IP_3 and the other sensitive to thapsigargin.

In the pharmacological characterization two types of high voltage-activated Ca^{2+} channels in *Xenopus laevis* melanotropes have been elucidated: the N-type and the L-type Ca^{2+} channel (Chapters 1 and 5). We show that, in contrast to the N-type channel, the L-type channel is not directly regulated by the secreto- inhibitors. After inhibition of both the N-type and the L-type channel, a Ca^{2+} current is still present, suggesting the presence of another type of Ca^{2+} channel (Chapter 1). Recent studies in mammalian brain tissues suggest the presence of other Ca^{2+} channels, all channels belonging to a superfamily of channel encoding genes [7]. Pharmacology and electrophysiology made clear that at least four additional high voltage-activated Ca^{2+} channels exist, that have been named O-, P-, Q- and R-type [8]. To date the presence of these types of Ca^{2+} channels in *Xenopus* melanotropes is under investigation.

Conclusion

In conclusion, Ca^{2+} oscillations in melanotrope cells of *Xenopus laevis* are initiated at the membrane by an N-type Ca^{2+} channel. It is here that secreto-inhibitors and secreto-stimulators can regulate the initiation and frequency of Ca^{2+} oscillations either through action on adenylyl cyclase or by affecting membrane potential. From the presented studies it is clear that secreto-inhibitors can have differential effects on the N-type channel. A challenging suggestion is that the animal can differentially release its inhibitory neurotransmitters, thereby selectively activating stronger or weaker inhibition mechanisms. The Ca^{2+} signal generated by the N-type channel is propagated to the nucleus through a process of CICR. The functional significance of the Ca^{2+} signal in the nucleus is unknown for *Xenopus* melanotropes. Future research within the department will be concerned with elucidating the relation between Ca^{2+} oscillations and biosynthesis activity in *Xenopus* melanotropes.

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SAMENVATTING

Voor het goed functioneren van een multicellulair organisme is een intensieve communicatie tussen de constituerende cellen van essentieel belang. Communicatie gebeurt in eerste instantie door cellulaire afgifte (secretie) van signaalmoleculen, de zogenaamde eerste boodschappers. Deze boodschappers binden vervolgens aan receptoren van de ontvangende cel. Na binding genereert deze cel een intracellulaire respons waardoor een of meerdere processen in deze cel worden gereguleerd. Deze intracellulaire signaaltransductie kan het gevolg zijn van ionkanalen in de celmembraan die de potentiaal van deze membraan veranderen, of van membraangebonden eiwitten of G-eiwitten die vervolgens kanalen activeren of inactiveren. Een andere mogelijkheid is dat de aan de membraan gebonden eiwitten enzymen activeren die op hun beurt weer boodschappermoleculen produceren, de zogenaamde tweede boodschappers. Er is de laatste jaren veel onderzoek verricht naar de rol van het calcium ion in de regulatie van verscheidene subcellulaire processen. Al in het begin van de jaren zestig werd gevonden dat een verhoging van de intracellulaire Ca^{2+} concentraties tot een verhoogde aanmaak van signaalmoleculen kan leiden. Tegenwoordig is het algemeen geaccepteerd dat Ca^{2+} noodzakelijk is voor allerlei cellulaire processen, zoals secretie en genexpressie. In deze thesis is getracht meer inzicht te verkrijgen in de dynamiek van intracellulair Ca^{2+}, met bijzondere aandacht voor het secretieproces. Hiertoe is onderzoek verricht aan de melanotrope cellen van de Zuidafrikaanse klauwpad *Xenopus laevis*.

De Zuidafrikaanse klauwpad kan de kleurintensiteit van zijn huid aanpassen aan de lichtintensiteit van de omgeving waarin hij zich bevindt. Bij dit achtergrondadaptatieproces spelen de melanotrope cellen in de *pars intermedia* van de hypofyse een belangrijke rol. Deze cellen produceren het melanoforen-stimulerend hormoon, α-MSH. Dit hormoon zorgt voor de spreiding van het zwarte pigment melanine in huidpigmentcellen, de melanoforen. In een donkere omgeving geven melanotrope cellen van de klauwpad veel α-MSH af terwijl ze op een lichte achtergrond juist inactief zijn. Voor een adequate regulatie van zijn huidskleur moet het dier derhalve beschikken over een goede regulatie van de α-MSH afgifte. Van de melanotrope cellen is bekend dat remming van de α-MSH afgifte gebeurt door de neuronale stoffen dopamine, neuropeptide Y en γ-aminoboterzuur (GABA). De melanotrope cel bezit twee typen receptor voor GABA: een $GABA_A$ receptor en een $GABA_B$ receptor. De $GABA_A$ receptor is een ionkanaal dat specifiek is voor chloorionen. Activatie van deze receptor leidt hoogstwaarschijnlijk tot een instroom van chloorionen waardoor de membraanpotentiaal sterker negatief wordt en de cel inactief wordt. De $GABA_B$ receptor bindt, evenals de dopamine receptor, aan een

G-eiwit, hetgeen leidt tot een verminderde productie van de tweede boodschapper cyclisch-adenosine monofosfaat (c-AMP). Dit heeft vervolgens weer een verminderde cellulaire activiteit tot gevolg. Stimulatie van de afgifte geschiedt onder invloed van het 'corticotropin-releasing hormone' (CRH) en het 'thyrotropin-releasing hormone' (TRH). Activatie van de CRH receptor stimuleert G-eiwitten. Deze G-eiwitten behoren echter tot een andere groep dan de G-eiwitten die geactiveerd worden door GABA en dopamine, en ze zorgen voor een toename van de c-AMP productie. In melanotrope cellen van de klauwpad is niet bekend welke tweede boodschappers worden geactiveerd na activatie van de TRH receptoren, maar activatie van deze receptor in een verwante amfibie, de groene kikker, *Rana ridibunda*, leidt tot toename van de intracellulaire inositol-1,4,5-trisfosfaat (IP_3) concentratie en stimulatie van cellulaire activiteit. Alhoewel er dus relatief veel bekend is over de rol van tweede boodschappers in de regulatie van celactiviteit bestaat er weinig inzicht in de rol die Ca^{2+} ionen in dit proces spelen.

Zoals boven al werd aangeduid, is voor een activatie van cellulaire processen zoals α-MSH secretie een toename van de cellulaire Ca^{2+} concentratie noodzakelijk. In theorie zijn er twee mogelijkheden die tot een dergelijke toename kunnen leiden. De eerste is een instroom van Ca^{2+} uit de extracellulaire ruimte. De tweede is het vrijmaken van Ca^{2+} uit intracellulaire opslagplaatsen zoals het endoplasmatisch of het sarcoplasmatisch reticulum. Instroom van Ca^{2+} uit de extracelullaire ruimte geschiedt wanneer Ca^{2+} kanalen openen door verandering van de membraan-potentiaal of door binding van eerste of tweede boodschappermoleculen aan receptoren die met deze kanalen in verbinding staan. Voor het vrijmaken van Ca^{2+} uit intracellulaire opslagplaatsen zijn de tweede boodschapper IP_3 en intracellulair Ca^{2+} veelal verantwoordelijk. In dit laatste geval spreken we van een door Ca^{2+} geïnduceerde Ca^{2+} afgifte (CICR). In veel cellen is de verhoging van de intracellulaire Ca^{2+} concentraties niet langdurig maar periodiek en repetitief, de zogenaamde Ca^{2+} oscillaties. Dit laatste geldt ook voor de melanotrope cel van *Xenopus*.

Welk van deze twee veranderingen in de Ca^{2+} dynamiek, de periodieke oscillaties of een langdurige verhoging, veranwoordelijk is voor de secretie is nog niet bekend. Beantwoording van deze vraag staat centraal in dit proefschrift.

Om inzicht te krijgen in de relatie tussen Ca^{2+} instroom mechanismen en hormoon afgifte zijn in Hoofdstuk 1 studies verricht naar de aanwezigheid van Ca^{2+}-kanalen en hun rol in het afgifteproces. Het blijkt dat melanotrope cellen een spontane electrische activiteit vertonen die zich uit in membraandepolarisaties. Tevens zijn twee typen Ca^{2+} kanalen aangetoond. Van deze twee typen is alleen het

zogenaamde N-type kanaal betrokken bij de Ca^{2+} instroom die noodzakelijk is voor het afgifte proces. Geïsoleerde melanotrope cellen werden gebruikt om de dynamiek van het intracellulaire Ca^{2+} te bestuderen (Hoofdstuk 2). Het bleek dat een overgrote meerderheid van de melanotrope cellen spontane Ca^{2+} oscillaties vertoont. Deze oscillaties werden geïnitieerd door een instroom van extracellulair Ca^{2+} via N-type kanalen. De in Hoofdstuk 3 en 4 beschreven experimenten werden uitgevoerd om een mogelijke rol van de spontane Ca^{2+} oscillaties bij de hormoonafgifte vast te stellen. In Hoofdstuk 3 is een overzicht gegeven van de effecten van afgifte-remmende en -stimulerende stoffen op de Ca^{2+} oscillaties. In Hoofdstuk 4 wordt een gedetaileerde studie naar het werkingsmechanisme van NPY gepresenteerd. Op basis van deze resultaten is geconcludeerd dat Ca^{2+} oscillaties, en niet de langdurige verhogingen van de intracellulaire Ca^{2+} concentratie, noodzakelijk zijn voor inductie van de afgifte van α-MSH. De werkingsmechanismen van de verschillende afgifte-remmende stoffen zijn onderzocht in Hoofdstuk 5. De verschillende stoffen blijken hun remming via verschillende receptorwerkingen te bewerkstelligen. De twee extremen zijn de $GABA_B$ receptor, die een gemakkelijk omkeerbare remming veroorzaakt, en de dopamine receptor, die een moeilijk omkeerbare remming teweeg brengt. In Hoofdstuk 6 zijn aspecten van Ca^{2+} oscillaties in tijd en ruimte onderzocht. Er wordt bewijsvoering aangedragen voor de stelling dat Ca^{2+} oscillaties zowel in het cytoplasma als in de kern voorkomen. Voortstuwing van de Ca^{2+} verhoging door de cel tijdens een oscillatie wordt zeer waarschijnlijk mogelijk gemaakt door in de cel aanwezige IP_3 receptoren op intracellulaire opslagplaatsen en CICR. Ook het binnengaan van de oscillaties in de kern is een actief proces waarbij perinucleaire Ca^{2+} opslagplaatsen zijn betrokken, en speelt wellicht een rol in genactivatie.

De resultaten die in dit proefschrift gepresenteerd zijn hebben geleid tot het opstellen van een model (zie pagina 100, Fig. 1) dat verklaart hoe de Ca^{2+} oscillaties in de melanotrope cel van de klauwpad worden opgewekt en gereguleerd.

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CURRICULUM VITAE

Wim Scheenen werd geboren op vrijdag 8 april 1966 in Baexem. Na in 1984 zijn eindexamen Atheneum-β te heben gehaald aan de scholengemeenschap St. Ursula te Horn begon hij zijn studie biologie aan de Katholieke Universiteit in Nijmegen. In februari 1990 behaalde hij het doctoraal examen met als hoofdvakken psychoneurofarmacologie (onder leiding van Prof. dr. A.R. Cools) en dierfysiologie (onder leiding van Dr. B.G. Jenks). Vanaf september 1990 tot september 1994 was hij werkzaam bij de Vakgroep Experimentele Dierkunde van de Katholieke Universiteit in Nijmegen als Onderzoeker in Opleiding, binnen het NWO onderzoeksthema "Neuropeptiden en Gedrag". In deze periode werd het onderzoek verricht dat in dit proefschrift is beschreven. Naast het verrichten van onderzoek werd een bijdrage geleverd aan het doctoraal onderwijs van biologie studenten. In de periode oktober 1990 tot december 1994 is Wim actief geweest binnen de jongeren afdeling van het NIBI, waar hij van februari 1993 tot december 1994 als voorzitter heeft gediend. Thans is de schrijver van dit proefschrift werkzaam als post-doctoraal medewerker in het laboratorium van Prof. dr. T. Pozzan te Padova in het kader van een Europees HCM project.